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POLYUNSATURATED FATTY ACIDS IN TUMOUR-INDUCED CACHEXIA.

ELIZABETH ANN HUDSON.

Doctor of Philosophy.

THE UNIVERSITY OF ASTON IN BIRMINGHAM.

September 1993.

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THE UNIVERSITY OF ASTON IN BIRMINGHAM.

POLYUNSATURATED FATTY ACIDS IN TUMOUR-INDUCED CACHEXIA.

by

ELIZABETH ANN HUDSON.

Submitted for the degree of Doctor of Philosophy.

A transplantable murine colon adenocarcinoma (MAC16) was utilised as a model of human cancer cachexia. This tumour has been found to produce extensive weight loss, characterised by depletion of host body protein and lipid stores at a small tumour burden. This weight loss has been found to be associated with production by the tumour of a lipolytic factor, activity of which was inhibited *in vitro* by the polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA). EPA has also been shown to possess anti-tumour and anti-cachectic activity *in vivo*, leading to the hypothesis that fatty acids mobilised by the lipolytic factor supply a growth requirement of the MAC16 tumour.

In this study mobilisation and sequestration of fatty acids by the tumour was found to be non-specific, although a relationship between weight loss and arachidonic acid (AA) concentration was found in both tumour-bearing mice, and human cancer patients.

The anti-tumour effect of EPA, which was found to be associated with an increase in cell loss, but not its anti-cachectic activity, was reversed by the administration of the PUFAs oleic acid (OA) and linoleic acid (LA). LA was also found to be capable of stimulating tumour growth. Inhibition of either the cyclooxygenase or lipoxygenase pathways was found to result in reduction of tumour growth, leading to the implication of one of the metabolites of LA or AA in tumour growth and cachexia.

The ethyl ester of EPA was found to be inactive against the growth and cachexia of the MAC16 tumour, due to its retarded uptake compared with the free acid. The anti-proliferative agent 5-fluorouracil was found to cause tumour growth inhibition, and when given in combination with EPA, reduced the phase of tumour regrowth observed after 4 to 5 days of treatment with EPA.

Key Words.

Cancer cachexia, tumour growth, polyunsaturated fatty acids, eicosapentaenoic acid, MAC16 adenocarcinoma.

TO DAD

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SECTION 1.0 INTRODUCTION.

1.0 INTRODUCTION

Cancer, the uncontrolled growth and invasion of neoplastic tissue into surrounding organs, and the consequent metastasis to other sites, is the second most common cause of death in the developed world after cardiovascular disease (Murray, 1990). It is arguably the more debilitating and distressing of the two disease states; both lack of understanding, and fear of the uncontrolled growth making cancer one of the remaining taboos of Western society.

1.1 Causes of Death in Cancer Patients.

Causes of death in cancer patients are found to be almost as varied as the disease itself, 19 separate causes of death being recorded at post mortem of 500 patients involving 23 different sites of carcinoma (Warren, 1932). Some causes of death are particularly site specific, for example death from renal insufficiency occured in 13% of cases in the Warren study (1932). Of these cases 26% involved cancer of the bladder, 42% of the cervix, and 19% occurred in patients with prostate cancer. In these groups, renal insufficiency accounted for 74%, 42%, and 63% of deaths respectively.

There is one cause of death, however, which is found to occur throughout the spectrum of cancer, the condition known as cancer cachexia. Warren (1932) found cachexia to be the cause of death in over 22% of cancer patients, the highest incidence attributed to a single cause of death, exceeded only by the total of all pulmonary related disorders. Other studies have put the incidence of cachexia as a primary cause of death at 10% (Inagaki *et al.*, 1974) or as high as 67% (Harnett, 1952). It is almost certain that the incidence of cachexia in cancer patients is higher than reported in at least two of these studies, as the condition may well occur in patients without it being considered as the immediate cause of death (Warren, 1932).

1.2 Cancer Cachexia.

The difficulty in attributing numbers to the incidence of cancer cachexia can be appreciated when two definitions of the syndrome are considered :

firstly,

> "...a systemic derangement of host metabolism which results in progressive wasting"
>
> (Lindsey, 1986).

and secondly,

> "the sum of those effects produced by neoplasms in the host, which are not the immediate result of mechanical interference with recognizable structures".
>
> (Costa, 1963).

The breadth of these definitions reflects the variety of symptoms experienced by patients affected by the condition, and which culminate in the severe weight loss and general malaise widely associated with the progression of cancer. Indeed the very etymology of the word cachexia, from the Greek *kakos hexis* meaning simply "bad condition" (Shamberger, 1984), reflects the polymorphism of the state.

The predominant characteristic of cachexia is progressive, involuntary weight loss (Kern, 1988), commonly accompanied by anorexia and nausea (Shamberger, 1984). Other clinically recognised manifestations of cancer cachexia include weakness, muscle atrophy, easy fatigue, impaired immune function, decreased motor and mental skills, decline in attention span and concentration ability (Lindsey, 1986), electrolyte and water abnormalities (Costa, 1963), anaemia, elevated basal metabolic rate, malabsorption, and diarrhoea (Shamberger, 1984).

1.2.1 Weight Loss in Cancer Patients.

It has been suggested by Lindsey (1986) that within a cancer population, the elderly, with their already compromised nutrient assimilation, and children and adolescents in critical periods of growth, when undergoing aggressive anti-cancer treatments may be particularly vulnerable to cachexia.

A multitude of factors contribute to the weight loss experienced by cancer patients in the form of cachexia (Fig. 1). Onset of weight loss appears to be independent of tumour size, in some cases weight loss is itself the sole presenting factor, causing patients to seek medical attention long before detection of a cancerous growth. Extensive cachexia has been observed in cases where the tumour mass amounts to less than 0.01% of body weight, just a few grammes, whereas in other cases weight loss may be of late onset, as a result of a larger tumour volume and progression of the disease (Lindsey, 1986). The incidence of weight loss has been found to vary greatly however with tumour type, as has the frequency of severe weight loss (>10%) (Table 1.). This variation may reflect time of presentation for medical attention, for example in breast cancer and sarcoma (low frequency weight loss group), self-detection of the primary tumour due to its site is quite common allowing the possibility of early treatment. In contrast, pancreatic and gastric tumours (high frequency weight loss group) are not easily detected, and often are only diagnosed on the appearance of secondary symptoms (DeWys, 1986).

A correlation may exist between weight loss and extent of disease; breast cancer patients with metastases to two or more anatomical sites (bone, liver or lung) showed significant increase in the incidence of weight loss (49%) when compared with those with involvement of less that two sites (31%) (DeWys, 1986). Incidence of weight loss has also been found to correlate with performance status, those with favourable performance scores consistently having a lower frequency of weight loss than patients with poor scores (DeWys, 1986).

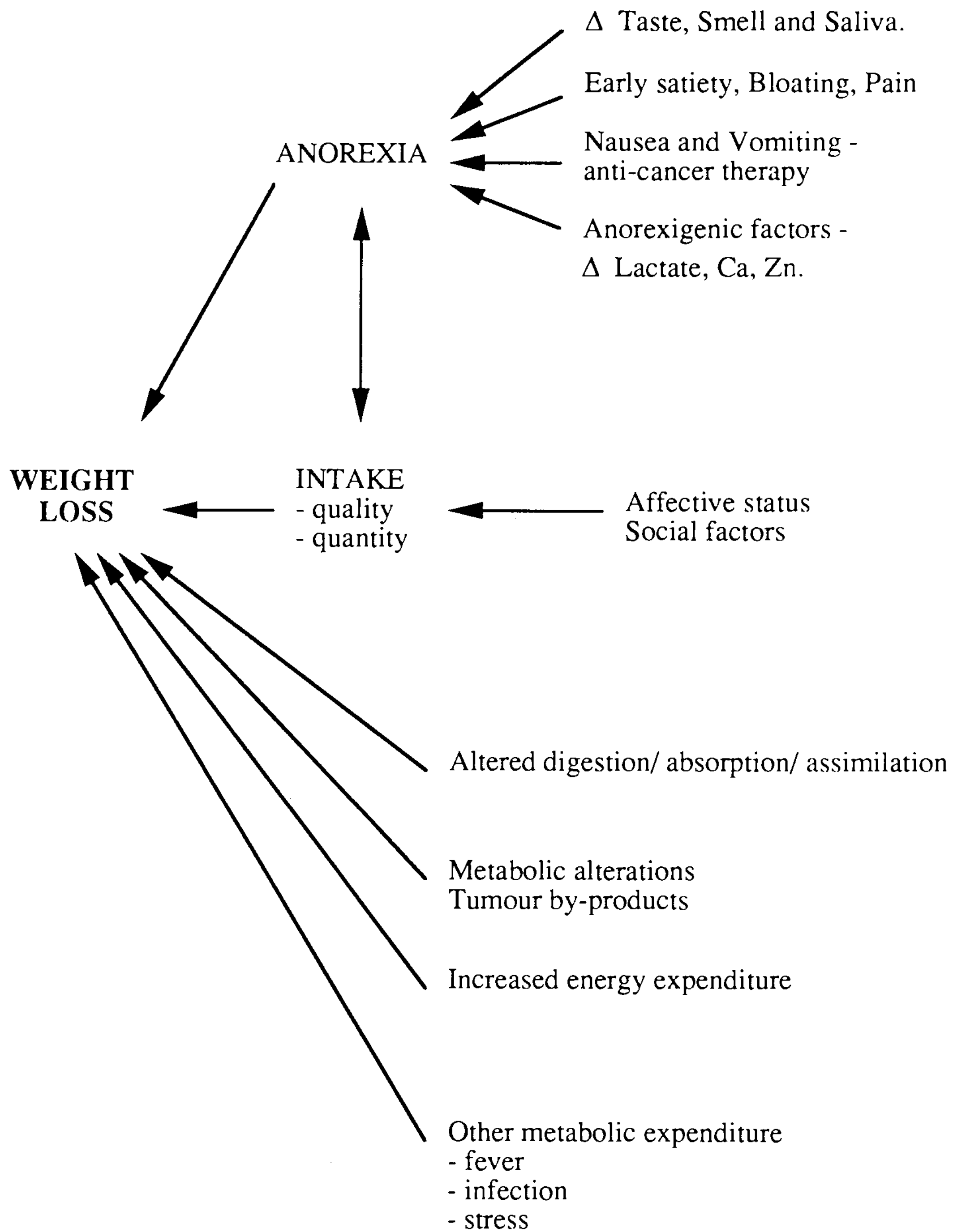

Figure 1. Factors affecting weight loss in cancer cachexia.
(From Lindsey, 1986).

Tumour Type	Incidence of Weight Loss	Incidence of Severe Weight Loss (>10%)
Favourable non-Hodgkin's lymphoma, Breast cancer, Acute non-lymphatic leukaemia, Sarcoma.	Low (31 - 40%)	4 - 10%
Unfavourable non-Hodgkin's lymphoma, Colon cancer, Prostatic cancer, Lung cancer.	Intermediate (48 - 61%)	10 - 15%
Pancreatic cancer, Gastric cancer	High (83 - 87%)	26 - 38%

Table 1. Frequency of weight loss in cancer patients.
(Data from DeWys, 1986).

Survival in all cases was found to be decreased in patients in which weight loss occurred, for both performance score and tumour extent, indicating that cachexia is a true indication of poor prognosis, a weight loss of as little as 5-6% being significantly deleterious to survival (DeWys, 1986; Heber and Tchekmedyian, 1992). A further disadvantage with regard to weight loss is response rate to chemotherapy; weight losing breast cancer patients were found to have a significantly lower response rate (44%) than patients who did not lose weight (61%). Weight loss in the cancer patient is for several reasons therefore, most undesirable; in conjunction with physical factors, there is also the psychological impact on the patient. Perception of this gradual wasting can lead to acknowledgment by the patient of their terminal state, resulting in apathy and a loss of enthusiasm for life (Lindsey, 1986).

There are numerous possible theories as to the mechanism of weight loss experienced by cancer patients; these can be broadly separated into three themes :

1). Weight loss due to "simple" starvation as a result of anorexia;
2). Weight loss due to perturbations in metabolic homeostasis caused by competition between host and tumour for energy and nutrient resources;
3). Production of cachectic factors by the tumour causing lipid mobilisation and breakdown of protein stores.

It is likely that the truth lies in a combination of these factors. Anorexia certainly occurs as a characteristic of cachexia in cancer patients without the two syndromes necessarily being concomitant. It has also been noted that the usual host adaptations to starvation or anorexia are not seen in cachectic cancer patients (Lindsey, 1986). Severe disruptions of host metabolism occur in many cancer patients, although direct competition for nutrients between host and tumour is unlikely to result in the extensive wasting observed in cachexia. Williams and Matthai (1981) cite the example of the much more rapid growth of a foetus, having little or no adverse effect on the mother, and compare this situation to that of the much smaller, yet deadly tumour burden in cachexia.

Several compounds capable of inducing lipid mobilisation or weight loss have been proposed as cachectic factors. Contention exists however over whether some of these compounds are in fact anorexigenic rather than cachexia-inducing, and work continues in this field. A fuller discussion of these theories appears in subsequent sections.

1.2.2 Contribution of Anorexia to Cachexia.

Anorexia, simply reduced food intake, is very commonly associated with cachexia (Shamberger, 1984); as well as greatly contributing to the weight loss of cachexia, anorexia may be one of the first major manifestations of the disease state of cancer (Schein *et al.*, 1975; Knox, 1983).

Many factors contribute to the anorexia of these patients (Fig. 1.), several of which are directly related to the anti-cancer treatment received (Lindsey, 1986). Virtually all anti-neoplastic drugs adversely affect food intake, many causing nausea and vomiting, for example nitrogen mustard, or an adriamycin/ cyclophosphamide combination, while others cause mucosistis, for example 5-fluorouracil and methotrexate. Other common side effect include ileus, hepatocellular damage and pancreatitis (Donaldson and Lenon, 1979).

Radiation therapy also produces anorexigenic side effects. Radiation to the abdomen and pelvis often causes severe sickness and diarrhoea accompanied by mucosal changes, ulceration and general enteritis (Donaldson, 1977). Therapy to the head and neck area may result in symptoms such as sore throat, dry mouth, mucosistis and ulceration, although of all symptoms it has been suggested that dulling of taste perception, "mouth blindness", has the most significant impact on the patient (Donaldson, 1977).

Abnormality in taste perception in cancer patients has been found to be commonly reported, most frequently as an increased threshold for sweetness, while other observed changes include decreased threshold for bitter, increased threshold for salt, and general decreased taste pleasurability. Patients may also experience

aversion to specific foodstuffs such as meat (DeWys, 1978; Schein *et al.*, 1975). Early satiety and decreased appetite can also increase anorexia; factors such as abnormal insulin response, increased levels of circulatory lipid and lactate, and other metabolic perturbations (section 1.2.3) observed in cancer patients may contribute to this response (DeWys, 1985). It has also been proposed that sustained stimulation of gastrointestinal receptors may occur in cancer patients (Knox, 1983), possibly due to mechanical obstruction by the tumour.

Theologides (1976) has suggested that production of peptide-like substances by the tumour and host may act directly on the sensor and responder cells of the hypothalamus and central nervous system, causing depression of appetite and therefore inducing anorexia. Other factors develop as a vicious circle induced by the anorexia and weight loss themselves. Weakness, tiredness, general malaise and psychological factors such as depression and apathy occurring as a result of cachexia in turn increase the anorexic component as even eating becomes a chore (Lindsey, 1986; Knox, 1983).

That cancer cachexia is not attributable to anorexia alone becomes clear when the differences in metabolism of the two conditions are considered (Fig. 2 and 3). Normal adaptations to starvation (section 1.2.2.1) result in a rate of weight loss which is much reduced when compared to that associated with major trauma, such as occurs in sepsis, burns or cancer cachexia (Brennan, 1977; Curreri and Luterman, 1978). Over a 30 day period, it has been estimated that (for an average 70kg male) chronic starvation may result in a total weight loss of 11%, compared with a possible 30% in severely traumatised patients (prolonged acute starvation can only be estimated to account for 22% weight loss over the same period). It has been suggested that weight loss in excess of 30% body weight is invariably fatal (Brennan, 1977).

Jeevanandam *et al.* (1984) have shown that in cachectic cancer patients, whole body protein turnover was 35% higher than in starved controls, and 32% higher than in non tumour-bearing weight losing patients. In another study, Moley *et al.* (1987) found that weight losing cancer patients demonstrated depletion of body cell mass in proportion to the degree of weight loss, while anorexia nervosa

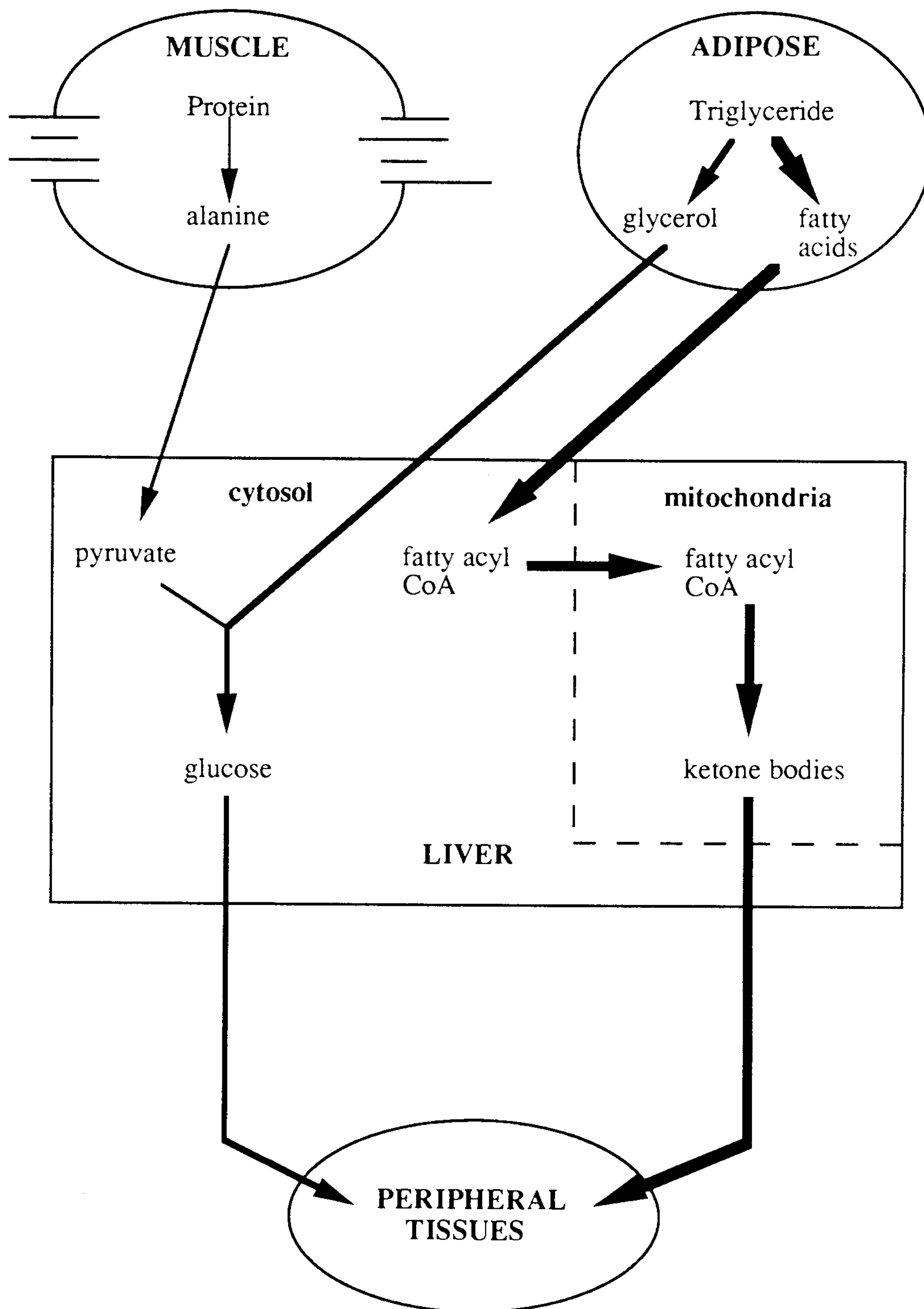

Figure 2. Metabolic adaptations to starvation.
(Adapted from Tisdale, 1986).

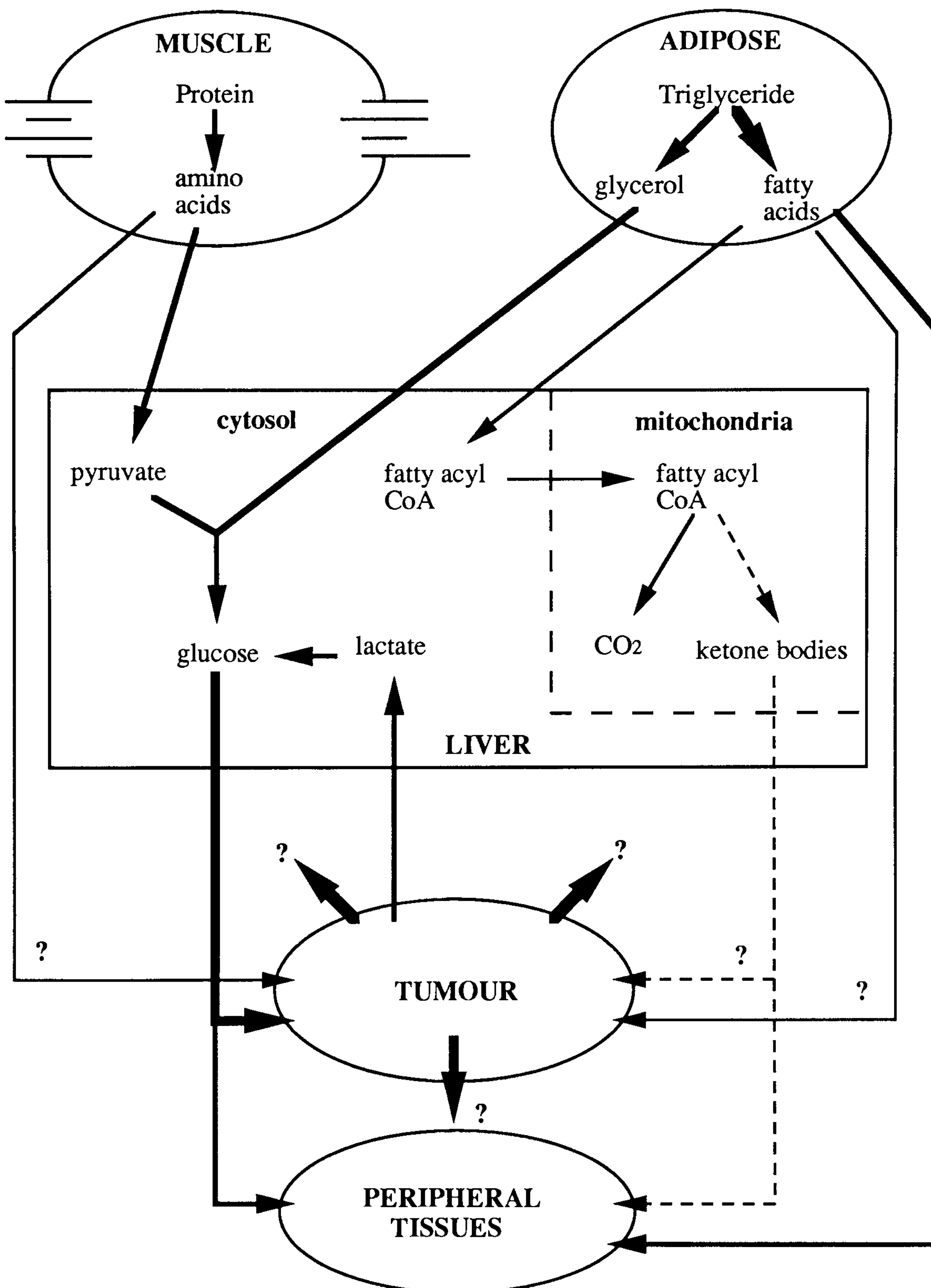

Figure 3. Metabolic pathways in cachexia.

patients showed sparing of body cell mass as a percentage of body weight despite an absolute decrease in mass, suggesting a preferential utilisation of fat as an energy source in these patients.

It is apparent, that the weight loss observed in cancer patients is different from that associated with other disease states. Anorexia is not sufficient to account for the metabolic abnormalities displayed by the cachectic cancer patient (section 1.2.3) and it is therefore important to consider anorexia as a contributory factor of cachexia, rather than as an explanation of the whole.

1.2.2.1 Metabolic Adaptations to Starvation.

During acute starvation, an increase in glucagon secretion and a decrease in insulin secretion occur in response to low blood sugar levels. Fluctuations in these hormones result in mobilisation of fatty acid stores, and decrease the uptake of glucose by muscles which therefore switch from glucose to fatty acid metabolism as a means of energy production (Voet and Voet, 1990). In an attempt to maintain a supply of glucose to the brain, gluconeogenesis from the degradation products of triglyceride and protein occurs in the liver. The accompanying production of urea results in a daily nitrogen loss of approximately 12g, equivalent to 75g muscle protein (320g wet muscle mass); at this rate, after 20 days 30% of muscle mass would be lost (Brennan, 1977).

A rapid adaptation to starvation occurs, with the production of ketone bodies (acetoacetate and β-hydroxybutyrate) from acetyl Coenzyme A in the liver mitochondria. (In mice, this adaptation has been found to occur after only 7 hours of starvation (Schreiber and Yeh, 1984)). The brain is able to metabolise ketone bodies as an energy source, thus greatly reducing the demand for glucose, and leading to conservation of protein as the burden of gluconeogenesis is relieved. This results in a fall in urinary nitrogen to 3g per day, allowing survival for long periods of time, with a loss of only 2% total body mass over 20 days in the state of chronic starvation (Brennan, 1977) (Fig. 2).

1.2.3 Host and Tumour Metabolism in Cancer Cachexia.

The relationship between tumour and host must be considered as one which is unique, although it can be most likened to that of a parasitic organism growing at the metabolic expense of its host. However, not only is the tumour capable of sequestering nutrients from the host circulation, but it may also release compounds into the circulation which confound host homeostasis thus exacerbating the metabolic disturbances observed as a result of tumour growth (Argiles and Azcon-Bieto, 1988). The metabolic processes associated with cachexia are summarised in Figure 3.

The establishment of the association between tumour and host is solely dependent on the development of a nutrient-carrying blood flow through the tumour. This vascularisation takes two forms; firstly, existing vessels undergo morphological and functional changes (venules become tortuous, elongated and dilated, and may eventually disintegrate or become blocked); secondly, later in development neovascularisation occurs as a result of the release by the tumour of angiogenesis factors (Vaupel *et al.*, 1989). Despite this, many tumours remain poorly vascularised resulting in large areas of hypoxic tissue.

1.2.3.1 Carbohydrate Metabolism and Energy Considerations.

It has been hypothesised that transformed cells undergo cell surface changes which result in altered membrane transport and an increased facility for the uptake of essential nutrients (Bhargava, 1977), this may lead to a preferential rate of glucose uptake by the tumour when compared with other host tissues. More recent evidence for the active transport of circulating glucose into the tumour has come from studies following the uptake of the glucose analogue ^{18}fluoro-2-deoxy-D-glucose into normal and neoplastic tissue; it has been found that uptake of this analogue into tumours of the lung was significantly increased when compared with uptake into the corresponding normal tissue (Nolop *et al.*, 1987).

This proposed channelling of glucose into the tumour may be reflected by a drop in blood glucose concentrations of the host, an occurrence which has been widely observed in patients with cancer of virtually every type (Kahn, 1980). Hypoglycaemia has also been demonstrated *in vivo* (Shapot and Blinov, 1974; Lundholm *et al.*, 1980), and in some cases has been found to correlate with tumour growth (Roh *et al.*, 1984), or with weight loss in cachectic mice (McDevitt and Tisdale, 1992), although in other studies a change in blood glucose has not been observed (Argiles and Lopez-Soriano, 1991).

Three possible mechanisms have been proposed to explain this hypoglycaemia; firstly, as already mentioned, a drain may occur on host circulation to supply the tumour requirement; secondly, a failure of homeostatic mechanisms (liver destruction, regulatory hormone suppression, inhibition of glucose storage mechanisms) may occur, or thirdly, the secretion of insulin or an insulin-like substance (such as IGF I and IGF II) may act to lower basal circulatory levels of glucose (Kahn, 1980).

Cancer patients frequently exhibit glucose intolerance, characterised by delayed clearance of blood glucose following either oral or intra-venous administration, resulting in a temporary hyperglycaemic state. It has been speculated that this may be due to a decrease in receptor affinity for insulin (Kisner *et al.*, 1978), possibly in combination with a reduced insulin secretion in response to the hyperglycaemia (Holroyde and Reichard, 1981), although it is far from clear at this time exactly what the relationship is between tumour growth, insulin production and insulin receptor sensitivity.

In 1930 Warburg and co-workers published a series of papers which demonstrated that in tumour tissue, not only does glycolysis occur, but that it is the dominant process of glucose metabolism, the ratio of sugar decomposition to oxidation being calculated as 12:1. Normally under aerobic conditions, glycolysis would be virtually replaced by the process of respiration in tissues.

The high rate of glycolysis which has been found in many tumours may result in an increased concentration of pyruvate in tumour cells, this is accompanied by an increase in the cytosolic NADH/ NAD^+ ratio, thus favouring the reduction of

pyruvate to lactate via the action of lactate dehydrogenase (Argiles and Azcon-Bieto, 1988). High levels of lactate dehydrogenase activity have also been found in tumour cells, resulting in an increase in the ratio of lactate dehydrogenase/ pyruvate dehydrogenase and further favouring the conversion of pyruvate to lactate rather than to acetyl Coenzyme A (Lazo and Sols, 1980). Poor tumour vascularisation leads to slow clearance of the lactate which then accumulates, causing a drop in the pH of the glycolytic tumour cells. This lowering in pH has been found to correlate with increasing tumour mass in the Walker-256 carcinoma and was shown to fall further after glucose loading (Jain *et al.*, 1984). Interestingly, acidosis is one factor which may both decrease the radiosensitivity of mammalian cells, and modulate the cytotoxicity of certain anti-cancer drugs (Vaupel *et al.*, 1989).

Lactate produced by the tumour in this way is transported to the liver where it enters the energy-expensive Cori cycle, being converted back to glucose via gluconeogenesis. It has however, been proposed that this is not a one way process, and that lactic acid is not only produced by tumours, but is also taken up by them from arterial blood in a two way flow, which may approach equilibrium (Sauer and Dauchy, 1986).

It is characteristic of many cachexia models that the host is found to have a vastly increased rate of gluconeogenesis from many precursors, such as glycerol (Lundholm *et al.*, 1982), alanine (Roh *et al.*,1984; Waterhouse *et al.*, 1979; Lundholm *et al.*, 1980) and lactate (Roh *et al.*, 1984). The liver of rats bearing the Walker-256 carcinoma has been found to contain elevated levels of certain intermediates of glucose metabolism, glucose-6-phosphate, fructose-1,6-bisphosphate and lactate, while others are decreased, glucose, pyruvate, and dihydroxyacetone phosphate, hexokinase activity has also been found to be increased (Argiles and Lopez-Soriano, 1991). Liver concentrations of citrate have also been found to be raised in both rats bearing the Walker-256, and mice bearing Sarcoma-180 tumours, leading to the suggestion that this may be yet another factor favouring gluconeogenesis, as citrate is capable of acting as an inhibitor of the glycolytic pathway enzyme, fructose bisphosphatase (McAllister *et al.*, 1982; Argiles and Lopez-Soriano, 1991).

Glycogen stores have been found to be depleted in the tumour-bearing state in both liver and muscle (Shapot and Blinov, 1974; Lundholm *et al.*, 1980). It has been proposed that this breakdown of glycogen, and increased rate of gluconeogenesis may occur in compensation for the hypoglycaemic tendencies exhibited by many hosts due to the glucose requirements of the tumour itself (Shapot and Blinov, 1974), thus leading to a constant state of glucose cycling, at the centre of which lies the Cori cycle.

Cori cycle activity has been found to be significantly increased in weight losing cancer patients (Holroyde *et al.*, 1975), a process which proves to be very energy expensive for the host. The metabolism by the tumour of glucose to lactate via glycolysis results in a net production of 2ATP per glucose molecule. Conversion of lactate to glucose via gluconeogenesis by the liver requires 6ATP per molecule of glucose produced, so therefore the host can be considered to have lost 8ATP for every molecule of glucose metabolised in this way (2ATP <u>to</u> the tumour, and 6ATP <u>from</u> the body) (Gold, 1968), regardless of the potential yield of ATP had the same glucose molecule been metabolised via a respiring system (38ATP/glucose) (Voet and Voet, 1990).

It was this observation which led Gold in 1968 to state that, "The metabolic implications of this situation are sufficient to account for the gradual weakening and ultimate debilitation so often seen in cancer patients ...." and that "this 'metabolic circuit' .... has every indication of being the ultimate thermodynamic means by which the body wastes and the cancer flourishes, ie. cachexia."

More recently Warnold *et al.* (1978) demonstrated that both daily energy expenditure, and resting metabolic rate were indeed significantly greater in cancer patients than in controls, thus supporting Gold's hypothesis. However, this theory does not account for the cachexia produced by a very small tumour burden, or for the lack of lactate metabolism and therefore Cori cycle activity found in some tumour lines (Aisenberg and Morris, 1961). A study by Mulligan and Tisdale (1991a), found that there was no significant difference between the glucose consumption of two histologically related murine adenocarcinomas, only one of

which induces cachexia in the host, suggesting that glucose consumption by the tumour is not sufficient alone to explain the wasting seen as a result of cancer cachexia.

1.2.3.2 Protein Metabolism.

Mider, in 1951 stated that "A progressively growing neoplasm, unlike the normal structures of the adult organism, increases its mass constantly through continuous manufacture of protoplasm. Anabolism exceeds catabolism." Mider (1951) went on to introduce the concept of the tumour as a nitrogen trap, proposing that the capacity of the tumour for protein synthesis is due to its pronounced avidity for amino acids, and suggesting that nitrogen lost from body tissues of cancer patients is translocated to the tumour.

Tumour cells have indeed been found to concentrate amino acids more efficiently than normal cells, RD3 sarcoma cells were able to selectively take up amino acids against a concentration gradient, indicating a mechanism for active uptake of amino acids into these cells (Wiseman and Ghadially, 1955). In the Lewis lung carcinoma, most amino acids were found in elevated concentrations in the tumour compared with liver or muscle (Rivera *et al.*, 1988), while plasma amino acid levels have been found to be decreased in cachectic animals bearing the MAC16 tumour (Beck and Tisdale, 1989a). Variations have been found in the amino acid composition of several tissues of tumour-bearing animals, such as plasma and brain (Krause *et al.*, 1979) and liver and muscle (Rivera *et al.*, 1988), and which when compared with pair-fed controls were found to be tumour related.

It has been proposed that the tumour may sequester specific amino acids from the host for its own requirements, causing an amino acid imbalance. Acids which are not taken up in this way may undergo transamination and deamination for channelling into the futile cycling of glucose via gluconeogenesis, thus providing a route for their elimination from the circulation (Stein, 1978). It is thought that glutamine in particular may be a favoured substrate for oxidation by the tumour; a

linear correlation has been found between tumour growth and intracellular concentration of glutamine, and a net flux towards the tumour was found to occur, suggesting a role for glutamine as a nitrogen donor (Carracosa *et al.*, 1984). The Lewis lung carcinoma has been found to oxidise glutamine in preference to alanine, glycine and glutamate whilst possessing high activity of the enzyme glutaminase (Rivera *et al.*, 1988). In contrast, a study by Kallinowski *et al.* (1987) found net glutamine uptake in only 13 of 65 human breast cancer xenografts, while 40 of the 65 showed net release. Kallinowski *et al.* (1987) have also suggested that *in vivo*, glutamine metabolism may not be important in tumours, as "glutaminolysis" is an oxygen dependant process, and therefore cannot occur in hypoxic areas of tumours. A concentration gradient in response to tumour requirements has also been proposed for leucine (Lazo, 1981).

This sequestering of amino acids from the host circulation may lead to a drain on the host's protein reserves, resulting in the loss of body protein and negative nitrogen balance often observed in cancer patients (Clark and Goodlad, 1971; Tessitore *et al.*, 1987) and characterised by hypoalbuminaemia (Heber *et al.*, 1985). Whole body nitrogen in mice bearing a cachexia-inducing murine adenocarcinoma has been found to decrease with increasing weight loss, while the tumour nitrogen content was found to increase with increasing weight loss. This finding supports evidence for the role of direct competition for nitrogen between tumour and host in the development of cancer cachexia, although the hypothesis does not explain the growth of tumours which do not cause cachexia of the host (Smith and Tisdale, 1993).

Whole body protein turnover has been found to be significantly increased in children recently diagnosed as having leukaemia or lymphoma (Kien and Camitta, 1983) and in cachectic cancer patients with a range of solid tumours (Jeevanandam *et al.*, 1984). In both studies, this increase in protein turnover was also associated with increased whole body protein synthesis, and in the leukaemia study, also with increased protein degradation. Shaw *et al.* (1991) found in cachectic cancer patients a significant increase in whole body protein catabolism, accompanied by an increase in protein synthesis. The study concluded that cancer patients are actively losing

protein due to the increase in catabolism, which is only partially compensated for by the increase in protein synthesis. In contrast, Lundholm *et al.* (1978) found increased degradation with decreased protein synthesis in patients with a variety of solid tumours, and Emery *et al.* (1984a) found muscle protein synthesis to be decreased (>80%) in cancer patients without an increase in muscle proteolysis, while whole body rates of protein synthesis and degradation showed no variation from control levels.

Other studies have also found decreased protein synthesis in cachectic tumour-bearing mice; Emery *et al.* (1984b) found a decrease in both muscle (70%) and liver (40%), while Smith and Tisdale (1993) found decreased muscle protein synthesis (60%) accompanied by elevated protein degradation which was also found to increase at high levels of weight loss. Tessitore *et al.* (1987) have observed increased muscle degradation with no change in protein synthesis rate, liver protein degradation was also prominent although in latter stages, protein synthesis in the liver was elevated. It has been suggested that this discrepancy in observed rates of protein synthesis could be due to differences in the nutritional status (fed or fasting) of the subjects between the studies (Smith and Tisdale, 1993), possibly with the rate of protein synthesis increasing in response to feeding to a lesser extent in the tumour-bearing state (Emery *et al.*, 1984b).

1.2.3.3 Lipid Metabolism.

Cachexia is characterised not only by muscle wasting, but also by a massive depletion of adipose tissue in response to tumour growth (Mays, 1969). The loss, of mainly neutral lipids, has been found not to be reversible by administration of a high fat diet, and was not displayed by pair-fed control animals indicating that the decrease in total body lipids was not merely a result of undernutrition (Lundholm *et al.*, 1981). Costa and Holland (1965) have described a triphasic pattern of fat loss from cachectic mice bearing the Krebs-2 carcinoma; a period of acute fat loss was found to occur just as tumours became palpable, which was followed by a

prolonged lag phase in the lipid loss, despite continuous tumour growth and finally a further substantial loss of body fat in the premortal phase resulting in an overall loss of approximately 75% of body lipid content. Mice bearing Ehrlich ascites tumours were also found to mobilise adipose tissue at an early stage of tumour development, glycerol release being significantly increased when compared to control animals (Balint, 1991).

A reduction in body lipid has also been found to occur in weight losing patients with a variety of cancers, although a small group of patients with breast cancer were found to show higher values of body fat when compared to a matched standard population. It was suggested that this finding correlated well with current hypotheses on the relationship between breast cancer and nutrition, in particular with dietary fat intake (Jardine *et al.*, 1984).

This breakdown in lipid stores of the body may be reflected by the hyperlipidaemia which has been observed to occur in conjunction with tumour growth, for example in Walker-256 tumour-bearing rats (Frederick and Begg, 1956). In this study no correlation was found between serum fatty acid concentration and tumour growth, but appearance of the hyperlipidaemia was found to coincide with the disappearance of fat from adipose stores. Hypertriglyceridaemia has been reported in mice bearing the Ehrlich ascites tumour (Balint, 1991) and in rats bearing methyl cholanthracene induced tumours, in which plasma cholesterol levels were also found to be increased (Redgrave *et al.*, 1984). In contrast, Briddon *et al.* (1991) have found increasing hypotriglyceridaemia with increasing weight loss in mice bearing the MAC16 tumour. The importance of this is unknown, as hyperlipidaemia is not found to be a frequent occurrence in cachectic cancer patients (Lundholm *et al.*, 1981).

Together with increased mobilisation of fat reserves, disturbances in fat storage have also been observed, lipogenesis has been found to be decreased in a range of *in vivo* models, such as Ehrlich ascites carcinoma (Kannan *et al.*, 1980), preputial gland tumours (Thompson *et al.*, 1981) and in human cancer patients (Jeevanandam *et al.*, 1986). It has been suggested that this may in part be due to redirection of exogenous fatty acids to the tumour, Thompson *et al.* (1981) found

that lipoprotein lipase activity showed a decrease in adipose tissue in conjuncton with the appearance of activity in large tumours. Mulligan and Tisdale (1991b) however, reported increased rates of lipogenesis in kidney, liver and adipose tissues in mice bearing either the cachexia-inducing MAC16 tumour, or the related non-cachectic MAC13 tumour. Further studies with the MAC16 model found increasing levels of lipoprotein lipase in heart and adipose to a maximum at approximately 15% weight loss, after which there was a gradual decline in activity to below control levels. This peak in lipoprotein lipase activity was found to coincide with a transient peak in plasma free fatty acid concentrations (Briddon *et al.*, 1991).

It is not clear at present whether an exogenous supply of host fatty acids is necessary for tumour growth; the majority of such studies have been performed on mice bearing the Ehrlich ascites carcinoma. Kannan *et al.* (1980) have proposed that *de novo* synthesis may be as important a source of lipids for the tumour as host circulatory fatty acids; Baker *et al.* (1977) have found that fatty acids sequestered by the tumour account for less than 1% of the lipid disappearing from the circulation, and proposed that the Ehrlich ascites carcinoma posed little or no competition to the host for fatty acids, and that tumour utilisation of fatty acids was unlikely to account for the loss of fat observed in cancerous animals. In contrast, it has been suggested by Spector and Steinberg (1967) that *de novo* synthesis of fatty acids is not an important source of lipid for the tumour cell; furthermore, it was found that at least 40-50% of fatty acid flux in ascites fluid resulted from uptake by tumour cells (Spector, 1967). However, Spector (1967) has also observed that Ehrlich ascites cells grow in culture without fatty acid supplementation, suggesting that while under physiological conditions exogenous fatty acids may be an important source of fat, they are not essential for its growth.

Cholesterol, an essential component of all membranes is acquired by replicating cells either by endogenous synthesis, or by uptake of low density lipoproteins (LDL) from plasma (Voet and Voet, 1990). Lombardi *et al.* (1989) have found high levels of expression of LDL receptors in a wide range of tumour lines *in vivo*, suggesting that LDL are supplied to the tumour chiefly by the exogenous route.

There has also been speculation over whether lipid is important as an energy source in the tumour-bearing state. In 1980, Kitada *et al.* found mobilisation of fatty acids from host adipose stores in response to growth of a thymic lymphoma. The fatty acids were found to be translocated to other tissues, although no increase in oxidation occurred compared with fed controls; starved controls undergoing lipid mobilisation showed increased oxidation of fat as an energy source. Similarly, fatty acid oxidation has been found not to differ from control rates in mice bearing the Ehrlich ascites carcinoma (Ookhtens and Baker, 1982). Mulligan (1991) found a decreased oxidation of labelled palmitate and triolein in mice bearing the MAC13 adenocarcinoma which grows without the expression of cachexia, and an increased oxidation in cachectic MAC16 tumour-bearing mice when compared to non tumour-bearing controls indicating utilisation of fat as a fuel source by the MAC16.

Oxidation of both short and long chain fatty acids has been found to vary considerably with growth rate and degree of differentiation in the Morris hepatoma (Bloch-Frankenthal *et al.*, 1965). The slow growing "minimal deviation" hepatomas such as the 5123, 7793 and 7787 have been found to be able to oxidise fatty acids to acetoacetate at a rate comparable to that of normal liver, while intermediate (7288C) or fast growing hepatomas, the 3924A, 3683 and Novikoff, were found to display very low capacity for fatty acid oxidation. It has also been proposed that there is an inverse relationship between fatty acid oxidation and glycolysis in these tumours; glycolysis being dominant in the faster growing hepatomas (Bloch-Frankenthal *et al.*, 1965).

Fields *et al.*, in 1981, found normal fatty acid oxidation in the livers of rats bearing either the Morris hepatoma 7800, or the weight loss-inducing 7777 hepatoma in both the fed and fasted state, and similar activity was found in the 7800 tumour. The 7777 hepatoma on the other hand showed several abnormalities of lipid metabolism, including low levels of fatty acyl Coenzyme A and carnitine palmitoyltransferase; tumour homogenates were also found to be incapable of oxidising palmitate, suggesting that the 7777 is unable to oxidise fat. In contrast, Ehrlich ascites tumour cells have been found to be capable of long chain fatty acid oxidation (Medes and Weinhouse, 1958), although utilisation depended on the

availability of the preferred substrate, glucose. A similar result has been obtained for oxidation of short and medium chain fatty acids by L1210 cells, uptake was found to decrease in the presence of glucose, and increase with increasing chain length (Burns *et al.*, 1979).

Pyruvate dehydrogenase, the enzyme which converts pyruvate to acetyl CoA, has been described as a key enzyme in weight loss associated with tumour growth (Fields *et al.*, 1982). Oxidation of fatty acids leading to increased concentrations of acetyl CoA would normally result in inhibition of pyruvate dehydrogenase and therefore prevent energy production at the expense of protein and carbohydrate precursors of pyruvate. A tissue which is incapable of fatty acid oxidation will exhibit a high activity of pyruvate dehydrogenase, resulting in a loss of pyruvate and extensive depletion of both glycogen and protein leading to weight loss (Fields *et al.*, 1982). It has been hypothesised by Fields *et al.* (1982) that this may occur in the tumour-bearing state, where lack of fatty acid oxidation in certain tumours such as the 7777 hepatoma, may increase the breakdown of carbohydrate and protein stores in the host.

The role of ketone bodies in cachexia and the tumour-bearing state is unclear, ketosis and ketonurea have been reported to be rare occurrences in cancer patients (Conyers *et al.*, 1979) and there has been no evidence for ketonurea in rodent models (Mider, 1951). This would not appear to be due to any defect in host metabolism, as studies have shown that ketosis can be induced by starvation in the tumour-bearing state (Rofe *et al.*, 1986; Magee *et al.*, 1979).

There have been mixed findings with regard to the utilisation of ketone bodies by the tumour, which it would appear are due mainly to variations in the activity of key enzymes of ketone metabolism. Activity of β-hydroxybutyrate dehydrogenase has been found to be retained by liver tumours, although at a reduced level when compared with normal liver, and at a higher level in slow growing well differentiated tumours than in rapidly growing, poorly differentiated tumours (Ohe *et al.*, 1967). In support of this finding, homogenates of the poorly differentiated Morris hepatoma 7777 were found to be incapable of ketone body

metabolism and had less than 10% of the β-hydroxybutyrate dehydrogenase activity of liver; activity of the enzyme 3-keto-acid transferase was also found to be undetectable in this tumour (Fields *et al.*, 1981). Variation in the activity of the enzyme acetoacetate CoA transferase however, appears to be in direct contrast to that of β-hydroxybutyrate dehydrogenase in hepatomas, Fenselau *et al.* (1975) found that while levels of acetoacetate CoA transferase were low in peripheral tissues, substantial levels of activity were to be found in tumours; activity was also found to increase with growth rate, the 7288CTC hepatoma having activity comparable to that of rat skeletal muscle. It was concluded by Fenselau *et al.* (1975), based on this evidence that the faster growing hepatomas had adequate enzyme activity to efficiently metabolise ketone bodies.

Studies in a range of both murine and human tumours of peripheral tissues found that while β-hydroxybutyrate dehydrogenase levels were equivalent to those in normal tissue, levels of the enzymes acetoacetate Coenzyme A transferase and acetoacetyl Coenzyme A thiolase were very low, suggesting that tumours of non-hepatic origin may be incapable of ketone metabolism (Tisdale and Brennan, 1983).

Sauer and Dauchy (1983) have found that ketone bodies were metabolised by a range of tumours, including the Morris hepatoma 7777, when the host animals were in the fasted state, although it was also observed that the presence of a tumour decreased the ketogenic response of the host to starvation. It would appear therefore, that while some tumours are able to make use of ketone bodies as a metabolic substrate others are not, probably as a result of deficiencies in the activity of crucial enzymes.

1.2.4 Catabolic Factors Proposed as Mediators of Cancer Cachexia.

In 1972, Theologides hypothesised that the metabolic perturbation which occurs in the cancer patient, leading to the syndrome of cachexia, may be due to alterations in the activity of certain enzymes, the regulation of which could be influenced by low molecular weight metabolites produced by the tumour itself.

This theory is now widely accepted, and evidence for the humoral mediation of cachexia came from Norton *et al.* in 1985, when it was demonstrated by parabiosis of rats that when coupled to a cachectic tumour-bearing rat, the non tumour-bearing pair exhibits manifestations of cachexia (weight loss, anorexia and elevated blood urea nitrogen), in direct response to the tumour-bearing state of its pair.

A number of factors have recently been proposed as being associated with the occurrence of cancer cachexia, a brief discussion of which is given below.

1.2.4.1 The Role of Cytokines in Cachexia.

1.2.4.1.1 Tumour Necrosis Factor (TNF) / Cachectin.

TNF/ cachectin has been found to be strongly associated with the cachexia of Trypanosome infections (Cerami *et al.*, 1985), and has been implicated in the pathological effects of cancer (Beutler and Cerami, 1986). Genetically engineered TNF-producing cells (CHO/TNF 20) have been shown to cause severe emaciation and loss of body fat on intra-muscular injection into nude mice. Extensive muscle wasting and anorexia were also reported in animals with tumour burdens of less than 5% host weight, and TNF was found to be present in the circulation of mice bearing the CHO/TNF 20 tumour (Oliff *et al.*, 1987).

TNF has also been detected in the serum of cachectic rats bearing a transplantable methylcholanthrene-induced sarcoma (Stovroff *et al.*, 1989), serum TNF levels correlated with tumour burden, weight loss, and decreased food intake,

the cachexia found in this model was attributed to the presence of TNF.

TNF is not implicated in the cachexia of all animal tumour models however. It has not been found to be detectable in the serum of NMRI mice bearing a cachexia-inducing murine adenocarcinoma (MAC16) (Mahony *et al.*, 1988), or of CDF1 mice bearing a murine colon adenocarcinoma derived tumour (C-26.IVX.) (Strassman *et al.*, 1992). Weight loss induced by the administration of TNF to non tumour-bearing mice was found to be correlated with a decreased food and water intake (Mahony and Tisdale, 1988; Mahony *et al.*, 1988), whereas anorexia is not thought to play a role in the cachexia of either the MAC16 tumour (Beck and Tisdale, 1991; Mulligan *et al.*, 1992a), or the C-26.IVX. tumour (Strassman *et al.*, 1992), as both tumour lines have been found to induce weight loss without a decrease in caloric intake. Administration of anti-TNF antibodies to MAC16 tumour-bearing mice has been shown to have no effect on either weight loss or tumour growth, leading to the conclusion that TNF is not involved in the mechanism of weight loss in this model (Mulligan *et al.*, 1992).

The involvement of TNF in the cachexia of human cancers is equally speculative, TNF having been detected in the serum of some, but not all cancer patients (Fearon *et al.*, 1991); it would seem therefore that while TNF may be present, it is not an absolute requirement for the development of cancer cachexia in the human patient (Oliff, 1988).

1.2.4.1.2 Interleukin-6 (IL-6).

There has recently been mounting evidence to suggest a role for IL-6 as a cachectic factor, elevated serum IL-6 levels have been detected in a number of mouse tumour lines including B16 melanoma and MCA-induced sarcomas and adenocarcinomas, while IL-6 was found to be undetectable in the serum of non tumour-bearing animals (McIntosh *et al.*, 1989). IL-6 levels were also found to increase linearly with tumour burden. IL-6 has also been detected in the serum of CDF1 mice bearing the C-26.IVX. adenocarcinoma, and also *in vitro*, in the conditioned medium of the same cell line (Strassman *et al.*, 1992). In this study the

circulatory level of IL-6 was found to correlate with weight loss, and resection of the tumour resulted in weight gain and a reduction in IL-6 levels within 5 days. Treatment of mice with anti-IL-6 antibody was also found to reverse the cachexia associated with this tumour model.

Fearon *et al.*, (1990), in a study of cachectic cancer patients found significantly increased circulatory levels of IL-6 compared with control non tumour-bearing, weight stable subjects, which it was suggested may be associated with the acute-phase protein response seen in these patients. Persistence of the acute phase response has been linked to depletion of body cell mass due to anorexia and mobilisation of peripheral tissues (Kern and Norton, 1988).

It has been suggested by Strassman *et al.* (1992), that IL-6 may be a common mediator of cachexia in a large proportion if not all experimental tumour models. Opposing evidence however comes from work performed by Mulligan *et al.* (1992) in which it was found that there was no increase in the IL-6 serum levels of cachectic mice bearing the MAC16 adenocarcinoma when compared with either mice bearing the MAC13 tumour, which grows without the production of cachexia, or control non tumour-bearing mice. Levels of IL-6 were not found to vary with weight loss in the cachectic animals, indicating that IL-6 is not involved in the expression of cachexia seen in this model.

1.2.4.2 The Role of the Endotoxin Toxohormone-L in Cachexia.

In 1981, Masuno *et al.*, purified and characterised a lipolytic factor found in the ascites fluid of mice inoculated with Sarcoma 180 cells; lipolytic activity was not found in ascites fluid unrelated to cancer. The factor was termed Toxohormone-L and was characterised as an acidic protein with a molecular weight of 75KDa. Body triglyceride levels were found to decrease during the growth of the Sarcoma 180, and it was suggested that this may have occurred as a result of the lipolytic action of this factor on adipose stores. Other indices of cachexia were not investigated.

Toxohormone-L has also been isolated from the ascites fluid of patients with hepatoma and injection of the purified material into non tumour-bearing rats was found to cause a severe suppression of food and water intake for up to three days after administration (Masuno *et al.*, 1984). This pronounced anorexigenic effect, combined with lack of further data precludes at the present time the consideration of Toxohormone-L as a potential cachectic factor.

1.2.4.3 The Role of Lipid Mobilising Factors (LMFs) in Cachexia.

A lipid mobilising factor, which acts directly on adipocytes, resulting in the release of glycerol and free fatty acids has been reported to occur in extracts of thymic lymphoma tissue grown in AKR mice (Kitada *et al.*, 1982). The factor was found to be a protein with a molecular weight of less than 10KDa, which on injection into non tumour-bearing mice with ^{14}C-labelled adipose tissue was found to cause rapid metabolism of the lipid stores to $^{14}CO_2$.

Recently, a lipid mobilising factor has been detected in the tumour homogenate of cachectic NMRI mice bearing the MAC16 adenocarcinoma (Beck and Tisdale, 1987). Growth of the tumour *in vivo* correlates linearly with weight loss, and is also associated with a decrease in both adipose stores and muscle dry mass in the host. Lipolytic activity in the plasma of MAC16 tumour-bearing mice was found to be double that found in either control non tumour-bearing mice, or those bearing the MAC13 tumour which grows without the expression of cachexia, indicating that the MAC16 tumour produces a circulatory lipolytic factor. Proteolytic activity was also found to be significantly increased in the plasma of MAC16 tumour-bearing mice when compared with both control states (Beck and Tisdale, 1987). Injection of the semi-purified factor into NMRI mice bearing the MAC13 adenocarcinoma has been found to be associated with the onset of weight loss without a decrease in food or water intake, suggesting that this factor may be responsible for the cachexia exhibited by the MAC16 tumour (Beck and Tisdale, 1990a).

In a study of cachectic cancer patients (Groundwater *et al.*, 1990), it was found that cancer patients with weight loss exhibited significantly higher serum lipolytic activity than either weight stable cancer patients, or cachectic Alzheimer's disease patients, both of which were shown to have serum lipolytic activity similar to that of healthy controls. The lipolytic activity of urine of cachectic cancer patients was not found to be significantly different from that of weight stable cancer patients, although both groups had activity which was significantly raised compared with the other control groups. Effective treatment of the cachectic cancer patients with either chemotherapy or radiotherapy was shown to be associated with a decrease in the lipolytic activity of the serum of these patients (Beck *et al.*, 1990b).

Recently a lipid mobilising factor has been purified and partially characterised from both urine and serum of cachectic cancer patients (Tisdale *et al.*, unpublished results), the factor has been found to be identical to that produced by the MAC16 tumour.

1.3 Therapy of Cancer Cachexia.

Reversal of the weight loss experienced by patients with cachexia has been found to occur after successful treatment or removal of the tumour (Lindsey, 1986). It is evident however, that in the majority of cases, patients would benefit greatly from reversal of weight loss prior to, and during therapy.

A number of potential anti-cachectic regimes have been proposed, most of which however, have met with limited success in the clinic. The conventional approach to treatment of a malnourished patient involves some form of hyperalimentation such as parenteral (TPN) or enteral (EN) nutrition. Nutrition of this type usually results in weight stabilisation, or weight gain shortly after initiation of the treatment (Cohn *et al.*, 1982). Cancer patients however, have been found to show significantly less improvement than other patients (Nixon *et al.*, 1981), and any benefit to the cancer patient is found to be of short duration, weight loss resuming, often at an increased rate immediately that artificial nutrition is ended (Terepka and Waterhouse, 1956). Several studies have found the weight gain experienced by cancer patients in response to artificial nutrition, to be due to increases in body fat and water content, with relatively small increases in lean body mass (Terepka and Waterhouse, 1956; Cohn *et al.*, 1982).

It has been suggested, that aggresive artificial nutrition may act to the detrement of the cancer patient (Terepka and Waterhouse, 1956), possibly due to acceleration of tumour growth in response to additional nutrients (DeWys, 1985). Despite this, TPN has been found to be of benefit in reducing major surgical complications (Bozetti, 1991), and is of use in maintaining weight during radiation therapy and chemotherapy (Donaldson and Lenon, 1979; Donaldson, 1977).

A number of treatments have been suggested with the aim of the reversal of metabolic abnormalities experienced by cancer patients. Gold (1968) proposed inhibition of gluconeogenesis as a simple yet effective mechanism of inhibiting tumour growth by reducing the amount of glucose available to the tumour as an energy source. Hydrazine sulphate, an inhibitor of the gluconeogenic enzyme phosphoenol pyruvate carboxykinase, has been found to improve survival of cancer

patients in studies in both the USA and Soviet Union (Chlebowski, 1991). Diminished carbohydrate intake, and administration of hypoglycaemic agents were also proposed as means of limiting glucose availability to the tumour (Gold, 1968).

Administration of insulin to tumour-bearing rats was found to compensate for endogenous insulin resistance; increased muscle uptake of glucose and amino acids occurred without a concurrent increase in uptake by the tumour. Host weight and muscle mass were also preserved as a consequence of insulin therapy (Kern and Norton, 1987). Insulin was also found to be successful in reversing the cachexia associated with growth of the MAC16 tumour in mice (Beck and Tisdale, 1989b), carcass fat and muscle mass also being found to be preserved. Administration of insulin in this model was however found to be associated with a 50% increase in tumour growth; this stimulation was found to be reversible by continuous administration of sodium D(-)-3-hydroxybutyrate in drinking water which alone has no effect on weight loss or tumour growth.

As previously discussed, certain tumours have been found to be incapable of fat and ketone metabolism, relying heavily on glucose as an energy source. An alternative to Gold's approach (1968) of targeting glucose metabolism in the tumour, is the induction of systemic ketosis in the host. Ketosis has been found to decrease plasma levels of glucose, lactate and pyruvate (Fearon *et al.*, 1988) in cachectic cancer patients, although no change was observed in host nitrogen balance, or protein turnover rates.

Dietary induced ketosis in mice has also been found to be successful in reversing cachexia. Beck and Tisdale (1989a) showed reversal of cachexia and inhibition of tumour growth in mice bearing the MAC16 adenocarcinoma when fed an 80% medium chain triglyceride (MCT) diet, and reported that body fat and muscle mass were also preserved. Magee *et al.* (1979) found a decrease in the number of B16 melanoma metastases occurring in the lung in response to induced ketosis in the host, adding to the evidence that a high fat diet may be of benefit in the treatment of cachexia.

Buzby *et al.* (1980) have found that host benefit from TPN varied greatly according to the diet employed; a diet in which calories were derived mainly from

carbohydrate and amino acids, resulted in maximal host maintenance, but also had the effect of greatly stimulating tumour growth. In contrast, a diet poviding non-nitrogenous calories in the form of fat, was found to increase host maintenance without any consequent increase in tumour growth. In another study, Mares-Perlman *et al.* (1988) reported no benefit of high fat TPN (FTPN) over conventional TPN in the maintenance of rats bearing the Morris hepatoma 7777, and found no difference in tumour growth rates between the two diets. It was suggested that evidence was insufficient to justify treatment of patients with FTPN.

Hunter *et al.*, (1989) found that supplementation of standard TPN solution with branched chain amino acids (BCAA) (leucine, isoleucine and valine) resulted in improved protein utilisation in cachectic cancer patients. Administration of BCAAs to sarcoma-bearing rats has been found to prolong survival by 32%, while causing a reduction in tumour growth (Schaur *et al.*, 1980), indicating improved metabolic resistance to carcass protein depletion. BCAAs were also found to be of benefit in preventing post-operative catabolism and in maintenance of body nitrogen post trauma (Freund *et al.*, 1979).

A compound which has received a lot of attention recently is megestrol acetate. Heckmayr and Gatzemeier (1992) reported that 80% of patients receiving megestrol acetate experienced increased well being, improved appetite, and weight gain which was found to be dose dependent. Side effects were reported to be very infrequent. Caloric intake and nutritional status were found to be increased in a further study of patients receiving megestrol acetate by Bruera (1992), and Loprinzi *et al.* (1992) also reported improved appetite, and non-fluid weight gain in patients. A study by Beck and Tisdale (1990) found that in MAC16 tumour-bearing mice, megestrol acetate was effective in reversing cachexia, and in stimulating appetite; the increase in weight however, was found to be due primarily to an increase in body water content. Administration of megestrol acetate in this model was also found to be associated with increased tumour growth, possibly due to elevated plasma glucose levels (Beck and Tisdale, 1990).

A range of drugs have been used to stimulate appetite in the cancer patient in the hope of overcoming cachexia to some extent, or to at least improve the state of

mind and therefore quality of life of patients. The corticosteroid prednisolone was found to improve appetite and well being, although it had no effect on caloric intake or body weight (Chlebowski, 1991). Another corticosteroid, dexamethasone was found to transiently increase appetite and strength, while the cannabinoid dronabinol was found to improve both mood and appetite and reduce the rate of weight loss in patients without however, drastically impeding its course (Bruera, 1992). The drug metoclopramide, commonly used to relieve chemotherapy associated nausea in cancer patients, has been reported to be successful in alleviating abdominal fullness (Chlebowski, 1991).

Recently, it has been found that fish oil is capable of inhibiting growth of the MAC16 tumour *in vivo*, and of inhibiting the cachexia associated with this tumour (Tisdale and Dhesi, 1990). This reduction in weight loss was found to be associated with increased total body fat and muscle mass. The two main components of fish oil are the n-3 fatty acids eicosapentaenoic acid (EPA) (20:5) and docosahexaenoic acid (DHA) (22:6). In a comparison of these two constituents of fish oil, Tisdale and Beck (1991) reported that the anti-cachectic effect of fish oil was due to the presence of EPA, DHA having no effect on either tumour growth or cachexia.

EPA has been found to exert its effects rapidly, and without any toxic side effects; it is due to enter Cancer Research Campaign phase I clinical trials in a short time as both an anti-cachectic and anti-tumour agent.

1.4 Fatty Acids in Cancer.

A correlation between dietary fat intake and carcinogenesis is rapidly becoming defined, particularly with regard to breast and colorectal cancer (Karmali, 1985; Nicholson *et al.*, 1988). Evidence suggests that not only is total dietary fat important, but also that the type of fat (n-6 or n-3 series) may influence carcinogenesis.

Epidemiological studies have found that west European and North American women have a three to four times higher incidence of breast cancer than Asian or Japanese women. Western diets consist of approximately 20% fat by weight (40% calories) which is found to be five times higher than a traditional Japanese diet based on rice and seafood (Pritchard *et al.*, 1989). Similarly, Eskimo women with their n-3 fatty acid rich diet have in the past been found to have a low incidence of breast cancer; a recent rise in breast cancer incidence may be related to an increased consumption of dietary fat, particularly of n-6 fatty acids as a result of Western influence (Karmali, 1985).

Experimental studies *in vivo* have been found to support these observations. King *et al.* (1979) found growth rates of mammary tumours to be increased in mice fed a high fat, or a polyunsaturated fatty acid (PUFA) rich diet compared with those fed a low fat diet or a saturated fat diet. This study also reported that in mice on a low fat diet only 9% of tumours grew to over 1cm in diameter, compared with over 40% of tumours in the high fat groups over the same time period. It would appear, that dietary fat may be implicated in both promotion and initiation of mammary carcinogenesis (Welsch, 1987); tumour yield being increased by a high fat diet whether fed before, during or after exposure to a carcinogen.

Fatty acids may not however be responsible only for tumourigenesis. Over the last ten years, evidence has emerged to suggest that while certain fatty acids of the n-6 series are capable of promoting tumourigenesis, others, and PUFAs of the n-3 series may have anti-tumour properties. The effect of non essential PUFAs on tumour growth is uncertain.

Diets supplemented with corn oil, rich in linoleic acid (LA) (18:2, n-6) have been found to stimulate tumour growth (Abraham and Hillyard, 1983), and to enhance tumour development (Jurkowski and Cave, 1985). Linoleic acid has also been reported to stimulate tumour cell growth *in vitro* (Buckman *et al.*, 1991; Rose and Connolly, 1989; Hussey, 1993 unpublished results), while diets rich in evening primrose oil containing γ-linolenic acid (GLA) (18:3, n-6) have been found to result in reduced mammary tumour growth (Pritchard *et al.*, 1989).

Supplementation of diets with fish oil, rich in the n-3 PUFAs eicosapentaenoic acid (EPA) (20:5) and docosahexaenoic acid (DHA) (22:6), have also been found to be associated with inhibition of growth of a range of mammary tumours (Karmali *et al.*, 1984; Jurkowski and Cave, 1985; Kort *et al.*, 1987; Pritchard *et al.*, 1989), in a transplantable human prostatic tumour line (Karmali *et al.*, 1987) and in both induced and transplantable colon carcinomas (Reddy and Maruyama, 1986; Gabor and Abraham, 1986; Sakaguchi *et al.*, 1990; Tisdale and Dhesi, 1990). Inhibition of growth of a human lung carcinoma in nude mice was also found to occur in response to a diet supplemented with an EPA/ DHA concentrate (deBravo *et al.*, 1991).

As stated in section 1.3, fish oil has been reported to have anti-cachectic activity (Tisdale and Dhesi, 1990), the active ingredient of which was found by Tisdale and Beck (1991) to be EPA. Anti-cancer activity of EPA has also been demonstrated by Minoura *et al.* (1988).

Nicholson *et al.* (1988) have suggested four mechanisms by which dietary fat may influence tumourigenesis : firstly, it is possible that fatty acids may have a direct effect on cellular proliferation; secondly, that the effect may be mediated by suppression of the immune response; thirdly, that changes in fatty acid composition, and therefore structure of cell membranes may make them more susceptible to carcinogenic stimuli; and fourthly, that eicosanoid metabolites of n-6 fatty acids may be responsible for the effect.

Of these hypotheses, the latter has in recent years received the most attention, and roles for products of both cyclooxygenase and lipoxygenase

pathways in tumour growth have been proposed (Karmali, 1980; Tsukada *et al.*, 1986; Buckman *et al.*, 1991; Lee and Ip, 1992).

It would appear that the environment of tumour cells, with particular regard to fatty acid concentration, plays an important role in the regulation of tumour growth. The specificity of the stimulation of tumour growth by n-6 fatty acids suggests that the tumour may have a requirement for one or more fatty acids which it may obtain from the host. It is possible that such a requirement may be related to the dramatic mobilisation of host lipid stores observed in cancer cachexia.

A deeper understanding of the relationship between fatty acids and tumour growth would surely lead to opportunities for the development of new anti-cancer therapies.

1.5 Aims of the Investigation.

Cancer cachexia causes severe complications in the treatment of cancer patients. The weight loss associated with the condition weakens the patient both physically and mentally, considerably decreasing the quality of life experienced by these patients. Cachexia is characterised in particular by mobilisation of host lipid stores, leading to the hypothesis that the tumour may have a requirement for one or more specific fatty acids.

The murine colon adenocarcinoma, MAC16 has been extensively characterised (Mulligan, 1991; Beck, 1989; Bibby *et al.*, 1987). When transplanted in NMRI mice this tumour results in the onset of cachexia at a very low tumour burden and is therefore very representative of the human condition. The MAC16 also exerts its effects on the host without causing a decrease in food or water intake, the model therefore allows the study of cachexia without the added complication of anorexia. Recently, a component of fish oil, eicosapentaenoic acid (EPA) has been found to have both anti-cachectic and anti-tumour activity against the MAC16 model of cachexia (Tisdale and Beck, 1991).

The aim of this project is to utilise the MAC16 model *in vivo* to extend knowledge of the role of polyunsaturated fatty acids in tumour growth and cachexia.

Tumour requirements for specific fatty acids are investigated by analysis of changes in the fatty acid composition of tissues in response to tumour growth and weight loss, and also the changes occurring in response to both the stimulation and inhibition of tumour growth by the oral administration of polyunsaturated fatty acids (PUFAs). Further investigation into the mechanisms of the action of the specific PUFAs linoleic acid (LA) and eicosapentaenoic acid (EPA) on tumour growth is made using cell kinetic studies.

An assessment is also made of the efficacy of ethyl esters of EPA, an EPA/ 5-fluorouracil combination, and various cyclooxygenase and lipoxygenase pathway inhibitors as anti-cachectic treatments.

SECTION 2.0 MATERIALS.

2.0 MATERIALS.

The addresses of all suppliers listed below are given at the end of the chapter.

2.1 Animals.

Pure strain inbred female NMRI mice were obtained from the Aston University breeding colony.

2.2 Chemicals.

The following compounds were purchased from suppliers as shown :

Boron trifluoride methanol (14%)	Sigma Chemical Co.
Butylated hydroxytoluene	Sigma Chemical Co.
Chloroform	Fisons
Corn oil (PUFA 54%, saturated fat 12%)	Safeway
Dicalcium carbonate	Pilsburys Ltd.
Dicalcium phosphate	Pilsburys Ltd.
Dimethyl sulphoxide	Sigma Chemical Co.
4,7,10,13,16,19-Docosahexanoic acid (90% +)	Sigma Chemical Co.
5,8,11,14-Eicosatetraynoic acid	Semat Technology
5,8,11,14,17-Eicosapentaenoic acid (80%)	Silbury
5,8,11,14,17-Eicosapentaenoic acid ethyl ester	Pronova Biocare
Ethanol	Fisons
5-Fluorouracil	Sigma Chemical Co.
G.C. column packing	Supelco
GP 3% SP-2310/ 25 SP-2300	
100/ 200 Chromasorb WAW.	

Gas chromatography standards	Sigma Chemical Co.
- methyl esters of :	
Palmitic acid	
Stearic acid	
Oleic acid	
Linoleic acid	
α-Linolenic acid	
Octadecatetraenoic acid	
Arachidonic acid	
Eicosapentaenoic acid	
Docosahexaenoic acid	
Halothane	ICI Chemical Industries plc.
n-Hexane	Sigma Chemical Co.
Hydrochloric acid	Fisons
Indomethacin	Sigma Chemical Co.
^{125}I-5-Iodo-2'-deoxyuridine (2000 Ci/mmol)	Amersham International
Linoleic acid (95%)	Sigma Chemical Co.
Liquid paraffin	Fisons
Liquigen triglyceride emulsion	Scientific Hosp. Supplies Ltd.
Margaric acid	Sigma Chemical Co.
Methanol	Fisons
(L)-Methionine	Sigma Chemical Co.
Methylcellulose	Fluka Biochemicals
Methyl margaric acid	Sigma Chemical Co.
Multiparin (heparin) (1000 units/ml)	Park Veterinary Hospital
Pig liver esterase (200 units/mg protein)	Sigma Chemical Co.
Potassium iodide	Fisons
Potassium di-hydrogen orthophosphate	BDH
di-Potassium hydrogen orthophosphate	BDH
Rat and mouse breeding diet	Pilsburys Ltd.

Rodent 006 premix	Pilsburys Ltd.
Sodium chloride	Sigma Chemical Co.
Sodium hydroxide	Fisons
Soya (dehulled)	Pilsburys Ltd.
Oleic acid (95%)	Sigma Chemical Co.

The following compounds were kindly donated by the companies listed below :

BW A4C
BW B70C
Carboxymethylcellulose
by Dr. L.G. Garland, The Wellcome Foundation.

Eicosapentaenoic acid : (EPA 80%, DHA 10%)
(EPA 90%, DHA 0%)
by Dr. D. Horrobin, Scotia Pharmaceuticals.

Docosahexaenoic acid ethyl ester
Eicosapentaenoic acid ethyl ester
Olive oil ethyl ester
by Dr. E. Mirrin, National Institute for Health, U.S.A.

Human serum samples from cachectic pancreatic cancer patients were very kindly collected and donated by Mr. K. Fearon, Edinburgh University.

2.3 Gases.

The following gases were obtained from BOC. Ltd.

Air, Argon, Hydrogen, Nitrogen (oxygen free), Nitrous oxide, and Oxygen.

2.4 Medium Chain Triglyceride Diet.

Composition.

	(g)
Soya (dehulled)	410.0
Dicalcium phosphate	45.75
Rodent 006 premix	21.40
Dicalcium carbonate	9.40
Sodium chloride	6.70
(L)-Methionine	2.10
Liquigen triglyceride emulsion	495.00 ml

Liquigen triglyceride emulsion consists of 52% medium chain triglycerides which are comprised of the following saturated fatty acids :

C_6 (1.1%), C_8 (81.1%), C_{10} (15.7%), and C_{12} (2.1%).

2.5 Phosphate Buffer.

K_2HPO_4 (10 mM) was adjusted to pH 8.0 by the addition of KH_2PO_4 (10mM).

2.6 Suppliers' Addresses.

Amersham International plc., Amersham Place, Little Chalfont, Bucks. U.K.

Aston University, Aston Triangle, Aston St., Birmingham. U.K.

BDH Ltd., Fourways, Carlyon Industrial Estate, Atherstone, Warwicks. U.K.

BOC Ltd., Lower Walsall St., Wolverhampton, West Midlands. U.K.

Fisons, Bishop Meadow Rd., Loughborough, Leics. U.K.

Fluka Biochemicals Ltd., The Old Brickyard, New Rd., Gillingham, Dorset. U.K.

ICI Chemical Industry plc., Pharmaceutics Division, Macclesfield, Cheshire. U.K.

National Institute for Health, Bethesda, Maryland. U.S.A.

Park Veterinary Hospital, Sutton Coldfield, West Midlands. U.K.

Pilsburys Ltd., 88A MacDonald St., Birmingham. U.K.

Pronova Biocare, PO Box 2109, N-3202 Sandefjord, Norway.

Safeway, High St., Bearwood, Warley, West Midlands. U.K.

Scientific Hospital Supplies Ltd., 100 Wavertree Boulevard, Wavertree Technology Park, Liverpool. U.K.

Scotia Pharmaceuticals Ltd., Woodbridge Meadows, Guildford, Surrey, U.K.

Semat Technology, 1 Executive Park, Watfield Rd., St Albans, Herts. U.K.

Sigma Chemical Co., Fancy Rd., Poole, Dorset. U.K.

Silbury, Warwick, U.K.

Supelco, Supelchem UK Ltd., Shire Hill, Saffron Walden, Essex. U.K.

The Wellcome Foundation Ltd., The Wellcome Research Laboratories, Langley Court, South Eden Park Rd., Beckenham, Kent. U.K.

SECTION 3.0 METHODS.

3.0 METHODS.

3.1 Transplantation of Tumours.

Transplantation of both MAC16 and MAC26 adenocarcinomas was performed by Mr. M.P. Wynter, Aston University. Tumours were excised from donor animals and placed in sterile isotonic saline; fragments (1-2mm^3) were implanted subcutaneously by trocar into the right flank of recipient female (18-22g) or male (25-27g) NMRI mice. For the purposes of this study, MAC16 adenocarcinomas were transplanted into female mice, unless otherwise stated, and MAC26 adenocarcinomas into male mice. After transplantation mice were fed on rat and mouse breeding diet, and water *ad libitum*. Body weight was recorded regularly from the date of transplantation, and tumour volume recorded as the neoplasm became palpable. Tumours were measured by means of callipers and volume was calculated from the formula :

$$\text{volume} = \frac{\text{length} \times (\text{width})^2}{2}$$

Mice were sacrificed before weight loss exceeded 30% of their maximum previous body weight, or if the tumour became necrotic and ulcerative. During treatment, mice were sacrificed immediately on becoming moribund. Treatment of tumour-bearing mice was commenced when the tumour volume was in the range of 87.5-200 mm^3, and when animals were judged to be cachectic, that is, exhibit steady weight loss in excess of 5% of their maximum previous body weight.

3.2 Collection of Tissues for Fatty Acid Analysis by Gas Chromatography.

3.2.1 Body Organs.

Animals were sacrificed by cervical dislocation, and the required organs (adipose tissue, liver, or tumour), were dissected out immediately. For storage, organs were snap frozen in liquid nitrogen and maintained at -70°C.

3.2.2 Plasma Samples.

Blood was extracted from mice either by cardiac puncture with a heparinised syringe under anaesthesia using Boyle's apparatus and a halothane, oxygen and nitrous oxide mixture, or by tail bleeding. Samples were collected into heparinised microfuge tubes and maintained below 4°C. Butylated hydroxytoluene, 0.01 volumes (2% in 50% ethanol), was added to the plasma after extraction from blood by centrifugation at 15,000g for 5 min, and samples were stored under nitrogen at -20°C.

3.3 Extraction of Fatty Acids for Analysis by Gas Chromatography.

The extraction of fatty acids from body organs and food materials was based on the method of Folch *et al.* (1957). Material was homogenised in 10 volumes of chloroform:methanol (2:1 vol./vol.), to which 50μl internal standard margaric acid (MA) (1.0 mg/ml) and 0.01 volumes of 2.0% butylated hydroxytoluene in 50% ethanol were added. The mixture was separated into organic and non-organic phases by centrifugation after mixing with 0.2 volumes of distilled water and 1.0ml methanol. The organic, lipid containing phase was then aspirated and was saponified by the addition of 5.0% sodium hydroxide in 50.0% methanol (2.5ml) and heating at 100°C for 45 - 60 min under argon. After cooling, the solution was

acidified to pH 2.0 by the addition of concentrated hydrochloric acid, and the fatty acids were then methylated by heating at 80°C for 5 min in the presence of 14.0% boron trifluoride methanol (2.5ml). The fatty acids were extracted twice with hexane:chloroform (4:1 vol./vol., 2.5ml), reduced by rotary evaporation, dissolved in 200µl hexane and stored under argon at -70°C before analysis by gas chromatography.

Plasma/serum samples (mouse or human) containing 0.01 volumes butylated hydroxytoluene (2.0% in 50% ethanol) and 50µl margaric acid (1.0 mg/ml) were extracted by following the above procedure from the sodium hydroxide hydrolysis step.

3.4 Fatty Acid Analysis by Gas Chromatography.

The fatty acid methyl esters were analysed using a Pye Unicam gas-liquid series 204 chromatograph with a flame ionisation detector. The column (2m x 2mm internal diameter) was packed with GP 3% SP-2310/2% SP-2300 on 100/200 Chromasorb WAW, and was run under a temperature programme of a 2°C/min. increase from 150°C to 220°C, which was then maintained for 10 minutes. The nitrogen gas flow rate through the column was 25.0 ml/min., and the air and hydrogen were run at 0.5 kg/cm^2 and 1.1 kg/cm^2 respectively.

Fatty acid peaks were identified using authentic standards, and concentrations of fatty acids were calculated using methyl margaric acid (MeMA) (1.0 mg/ml) as an external standard, and the following equations :

Let $\mathbf{x}$ = any fatty acid extracted

Let Y = area of $\mathbf{x}$ corrected for 100% recovery.

$$Z = 0.25 \times \text{area under MeMA}$$

$$\% \text{ Recovery} = \frac{\text{area MA}}{Z} \times 100$$

$$Y = \frac{\text{area } x}{\% \text{ Recovery}} \times 100$$

$$\begin{array}{r}\text{Conc. } x \text{ (mg/ml)} \\ \text{of injection sol'n} \\ \text{(corrected for recovery)}\end{array} = \frac{Y}{\text{area MeMA}}$$

$$\begin{array}{r}\text{Total fatty acids} \\ \text{in starting material}\end{array} = \frac{\text{Conc. } x \text{ (mg/ml)}}{5}$$

$$\begin{array}{r}\text{Conc. } x \text{ (mg/g)} \\ \text{of tissue}\end{array} = \frac{\text{Total fatty acids}}{\text{weight of tissue (g)}}$$

3.5 Effect of Cachexia on Fatty Acid Composition of Adipose, Liver, Plasma and Tumour of MAC16 Tumour-bearing Mice.

Fatty acids were extracted from the tissues of both MAC16 tumour-bearing mice exhibiting varying degrees of weight loss, and control (non tumour-bearing) mice. Samples were analysed by gas chromatography, and both tissue fatty acid concentration and total fatty acid content were calculated using the internal standard.

Adipose tissue was extracted from male MAC16 tumour-bearing mice in the form of epididymal fat pads. Collection of all other tissues was made from female mice.

3.6 Oral Administration of Polyunsaturated Fatty Acids to MAC16 Tumour-bearing Mice - Effect on Fatty Acid Composition of Tumour, Liver and Plasma.

3.6.1 Administration of Eicosapentaenoic Acid (EPA), Docosahexaenoic Acid (DHA) and Linoleic Acid (LA).

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily by gavage either EPA (80%, 2.0 g/kg), DHA (90+%, 2.25 g/kg), LA (76.7%, 1.9 g/kg), or a combination of EPA (80%, 2.0 g/kg) and LA (76.7%, 1.9 g/kg); control tumour-bearing mice received 50μl water. Daily recordings were made of body weight and tumour volume, and of food and water intake. In each group n=6, mice were randomised according to tumour volume, and received rat and mouse breeding diet and water *ad libitum*. Treatments were terminated after 4-5 days, and tumour, liver and plasma samples were collected from each group for determination of fatty acid content.

3.6.2 Administration of Eicosapentaenoic Acid (EPA) and Pure Corn Oil (CO).

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily by gavage either EPA (90%, 2.25 g/kg), pure corn oil (containing 54% PUFA) (5.0 g/kg), or a combination of the two treatments; control tumour-bearing mice received 50μl water. Daily recordings were made of body weight and tumour volume. In each group n=6, mice were randomised according to tumour volume, and received rat and mouse breeding diet and water *ad libitum*. Treatments were terminated after 4-5 days, and tumour and plasma samples were collected from each group for determination of fatty acid content, the fatty acid composition of the corn oil was also determined.

3.6.3 Administration of Eicosapentaenoic Acid (EPA) and Oleic Acid (OA).

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily by gavage either EPA (90%, 2.25 g/kg), OA (95%, 2.0 g/kg) or a combination of the two treatments; oleic acid was diluted to 2.0 g/kg with liquid paraffin, two groups of control mice received either 50µl water or 50µl liquid paraffin. Daily recordings were made of body weight and tumour volume. In each group n=6, mice were randomised according to tumour volume, and received rat and mouse breeding diet and water *ad libitum*. Treatments were terminated after 4-5 days, and tumour and plasma samples were collected from each group for determination of fatty acid content.

3.7 Determination of Growth Kinetics of MAC16 and MAC26 Tumours - Effects of Eicosapentaenoic Acid (EPA) and Linoleic Acid (LA).

3.7.1 Kinetics of Eicosapentaenoic Acid-induced MAC16 Tumour Growth Inhibition.

The potential doubling time, and tumour cell loss were calculated according to the method of Gabor *et al.* (1985), and Gabor and Abraham (1986). Tumour-bearing mice were given 0.1% potassium iodide in drinking water, 4 days prior to an intraperitoneal injection of [^{125}I]-5-iodo-2'-deoxyuridine (sp. act. 2000 Ci/mmol) in 0.1ml sterile saline, from which time control mice received daily 50µl water by gavage, while the second group received EPA (80%, 2.0 g/kg) by gavage. After 2 hours, 4 mice from each group were sacrificed and then at 24 hourly intervals for a further 5 days, tumours were removed immediately and stored at -20°C for 24 hours before refrigeration. Weight loss and tumour volume were recorded daily.

To investigate the kinetics of the second phase of EPA-treated tumour

growth, a group of animals were dosed with EPA (80%, 2.0 g/kg) as above, before receiving the i.p. injection of [^{125}I]-5-iodo-2'-deoxyuridine on day 4 of treatment, from which time 4 mice were sacrificed at 24 hourly intervals, and tumours removed and stored as above.

To determine tumour cell DNA-radioactivity, tumours were minced into 1-2mm^3 pieces and acid soluble material was removed by washing (x3) in methanol:acetic acid (3:1 vol./vol.) over 72 hours. Radioactive content of the tumour pieces was then detected using a Packard Tri-carb gamma-scintillation spectrometer. The values for cpm/g of tumour were plotted on a semi-logarithmic scale, and the $t^1/_2$ of the decline in specific activity was determined. The mean doubling time of the tumours in each group was determined by plotting percent increase in tumour volume against time.

From this data, cell loss, ϕ, was calculated using the equation :

$$\phi = 1 - \frac{t_{1/2}}{T_D}$$

3.7.2 Kinetics of Linoleic Acid Reversal of Eicosapentaenoic Acid-induced MAC16 Tumour Growth Inhibition.

The kinetics of the effect of linoleic acid on MAC16 tumour growth, and its reversal of EPA-induced tumour growth inhibition were investigated by administering daily doses by gavage of either LA (76.7%, 1.9 g/kg) or a mixture of EPA (80%, 2.0 g/kg) and LA (76.7%, 1.9 g/kg), while following the protocol for determination of cell loss and potential doubling time outlined above.

3.7.3 Kinetics of Linoleic Acid-induced Stimulation of MAC26 Tumour Growth.

This work was performed in collaboration with Ms. H.J. Hussey, Aston University.

The kinetics of the effect of linoleic acid on MAC26 tumour growth were investigated by following the protocol outlined above. Tumour-bearing mice (approx. 13 days post-transplantation) were orally dosed with either 50µl water as controls, or with linoleic acid (95%, 2.37 g/kg), for four days before receiving the i.p. injection of [^{125}I]-5-iodo-2'-deoxyuridine. Data was collected as above.

3.8 Investigation of the Effect of Ethyl Esters of Eicosapentaenoic Acid and Docosahexaenoic Acid on Tumour Growth and Weight Loss.

3.8.1 Oral Administration of Eicosapentaenoic Acid Ethyl Ester (EEE) and Docosahexaenoic Acid Ethyl Ester (DEE).

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily by gavage, one of the following treatments: EPA (80%, 2.0 g/kg), EEE (2.5 g/kg), DHA (90+%, 2.25 g/kg), DEE (2.5 g/kg) or olive oil ethyl ester (2.5 g/kg); control tumour-bearing mice received 50µl water. In each group n=6, mice were randomised according to tumour volume, and received rat and mouse breeding diet and water *ad libitum*. Daily recordings were made of body weight and tumour volume. The treatments were terminated after 5 days.

3.8.2 Oral Administration of Eicosapentaenoic Acid Ethyl Ester (EEE) in Combination With a Medium Chain Triglyceride (MCT) Diet.

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily by gavage either EEE (2.5 g/kg) or EEE (2.5 g/kg) plus MCT diet; control tumour-bearing mice received either 50µl water plus MCT diet or water alone. In each group n=6 and mice were randomised according to tumour volume.

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On day 1 of treatment the supply of rat and mouse breeding diet was removed and was replaced with the MCT diet (*ad libitum.*) as appropriate; animals not receiving the MCT diet were maintained on the normal rat and mouse diet, the two diets were both isocaloric and isonitrogenous. Food and water intake along with body weight and tumour volume of all groups was recorded daily. Treatment occurred within 4 hours of the start of the dark cycle, to ensure that food was consumed over the period immediately before and after treatment. On day 6 of treatment, blood samples were obtained by cardiac puncture, and tumours were removed. Fatty acid composition of plasma and tumours was determined by gas chromatography.

3.8.3 Oral Administration of Eicosapentaenoic Acid Ethyl Ester (EEE) Pretreated With Esterase.

3.8.3.1 Pretreatment of EPA Ethyl Ester with Pig Liver Esterase.

EPA ethyl ester was incubated at 37°C for 24 hours under nitrogen with phosphate buffer (10mM, pH 8.0) (1:1 vol./vol.) containing pig liver esterase (120 units/100mg EEE). The ester was treated on a daily basis to minimise storage of the fatty acid in an aqueous environment.

The concentration of ethyl ester and free acid in the mixture after treatment with the enzyme was determined by gas chromatography.

3.8.3.2 Treatment of MAC16 Tumour-bearing Mice.

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily by gavage either pretreated EEE/phosphate buffer esterase mixture (100μl) or EEE/phosphate buffer (1:1 vol./vol) (100μl); control tumour-bearing mice received phosphate buffer (10mM, pH 8.0) containing 7mg ethanol and 60 units esterase (50μl). Mice were randomised according to tumour volume (n=6),

and body weight and tumour volume were recorded daily. Mice received rat and mouse breeding diet and water *ad libitum*.

3.9 Pharmacokinetics of the Plasma Uptake of Eicosapentaenoic Acid (EPA) and its Ethyl Ester (EEE).

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily one of the following treatments: EPA (90%, 2.25 g/kg), EEE (2.5 g/kg), EEE (2.5 g/kg) plus MCT diet (section 3.8.2), or EEE pretreated with esterase (section 3.8.3); control tumour-bearing mice received either water plus MCT diet or water alone (50μl). In all groups n=6 and mice were randomised according to tumour volume. Body weight and tumour volume were recorded daily, mice received rat and mouse breeding diet and water *ad libitum* unless otherwise stated and treatments were terminated after 4 days.

Blood samples were collected by tail bleeding from mice in all groups at intervals over a 72 hour period, and both plasma and tumour eicosapentaenoic acid concentration was determined by gas chromatography.

3.10 Administration of 5-Fluorouracil (5-FU) in Combination with Eicosapentaenoic Acid (EPA) - Effect on Weight Loss and Tumour Growth.

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received one of the following treatments: EPA (90%, 2.25 g/kg), 5-FU (80.0, 100.0 mg/kg) or a combination of the two treatments. EPA was administered by gavage on a daily basis, while 5-FU was administered as an aqueous solution by i.p. injection (200μl) on alternate days from day 4 of the experiment. Two groups of mice received the combination treatment; in one group administration of EPA continued during 5-FU treatment, whereas the other group received EPA from days

1-4 only. Control tumour-bearing mice received water, either daily by gavage (50μl), or on alternate days from day 4 by i.p.injection (200μl). In all groups n=6, mice were randomised according to tumour volume and received rat and mouse breeding diet and water *ad libitum*. Body weight and tumour volume were recorded daily.

3.11 Administration of Cyclooxygenase and Lipoxygenase Inhibitors to MAC16 Tumour-bearing Mice - Effect on Weight Loss and Tumour Growth.

3.11.1 Oral Delivery of the Compounds BW A4C, Eicosatetraynoic Acid (ETYA), and Indomethacin in 1.0% Methylcellulose.

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily by gavage, one of the following treatments: BW A4C (5.0, 10.0 mg/kg), ETYA (5.0, 10.0 mg/kg), or indomethacin (2.5, 5.0 mg/kg); compounds were suspended in 1.0% methylcellulose by sonication. Control tumour-bearing mice received either water (100μl) or 1.0% methylcellulose (100μl). In all groups n=6, mice were randomised according to tumour volume and received rat and mouse breeding diet and water *ad libitum*. Daily recordings were made of body weight and tumour volume.

3.11.2 Oral Delivery of the Compounds BW A4C, BW B70C, and Indomethacin in 0.25% Carboxymethylcellulose.

Cachectic tumour-bearing mice (approx. 14 days post-transplantation) received daily by gavage either BW A4C (5.0, 10.0, 15.0 mg/kg), BW B70C (5.0, 10.0, 15.0 mg/kg) or indomethacin (2.5, 5.0 mg/kg); compounds were suspended in 0.25% carboxymethylcellulose by sonication. Control tumour-bearing mice

received either water (100μl) or 0.25% carboxymethylcellulose (100μl). In all groups n=6, mice were randomised according to tumour volume and received rat and mouse breeding diet *ad libitum*. Daily recordings were made of body weight and tumour volume.

3.11.3 Oral Delivery of the Compounds BW A4C, BW B70C and Indomethacin in 10.0% DMSO.

In a series of experiments cachectic tumour-bearing mice received daily by gavage one of the following treatments: BW A4C (1.0, 2.5, 5.0, 10.0, 20.0, 40.0 mg/kg), BW B70C (10.0 mg/kg), indomethacin (5.0, 10.0 mg/kg) or a combination of BW B70C (10.0 mg/kg) and indomethacin (5.0 mg/kg). Compounds were prepared as stock solutions in DMSO and were diluted to 10.0% with distilled water immediately before administration. Control tumour-bearing mice received either water (100μl) or 10.0% DMSO (100μl). In all groups n=6, mice were randomised according to tumour volume and received rat and mouse breeding diet and water *ad libitum*. Daily recordings were made of body weight and tumour volume.

SECTION 4.0 RESULTS AND DISCUSSION.

4.0 RESULTS AND DISCUSSION.

4.1 Effect of Weight Loss on the Fatty Acid Composition of Tissues of MAC16 Tumour-bearing Mice.

4.1.1 Introduction.

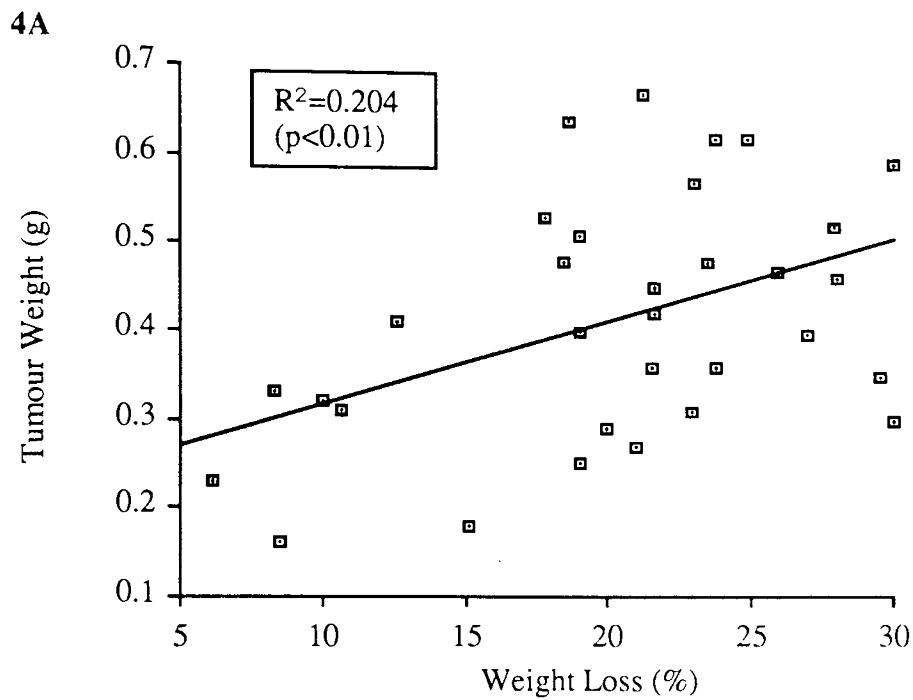
Cancer cachexia is characterised by severe depletion of adipose stores (Mays, 1969). As previously discussed, this mobilisation of lipid stores has been found to be associated with production by the tumour of a lipolytic factor both in human cancer patients (Groundwater *et al.*, 1990), and in the MAC16 *in vivo* model of cachexia (Beck and Tisdale, 1987). The inhibitory effect of EPA on the activity of the MAC16 tumour-derived lipolytic factor *in vitro*, combined with its known anti-tumour and anti-cachectic activity *in vivo* (Tisdale and Beck, 1991), suggests a relationship between the lipolysis occurring *in vivo* and tumour growth. Fatty acids released by the breakdown of adipose stores in cachexia may be being transported to the tumour to fulfill a specific growth requirement.

This study examines the fatty acid composition of adipose tissue, liver, plasma and tumours of MAC16 tumour-bearing mice with varying degrees of weight loss. Fatty acid composition of serum of weight losing cancer patients was also analysed.

4.1.2 Results

Weight loss of MAC16 tumour-bearing mice was found to correlate linearly with tumour growth in animals with a tumour burden of over 0.2g (approximately 1% body weight) (Fig.4A), while wet weight of both adipose tissue and liver were found to decrease with weight loss, and therefore tumour growth (Figs.4B and C).

4A



4B

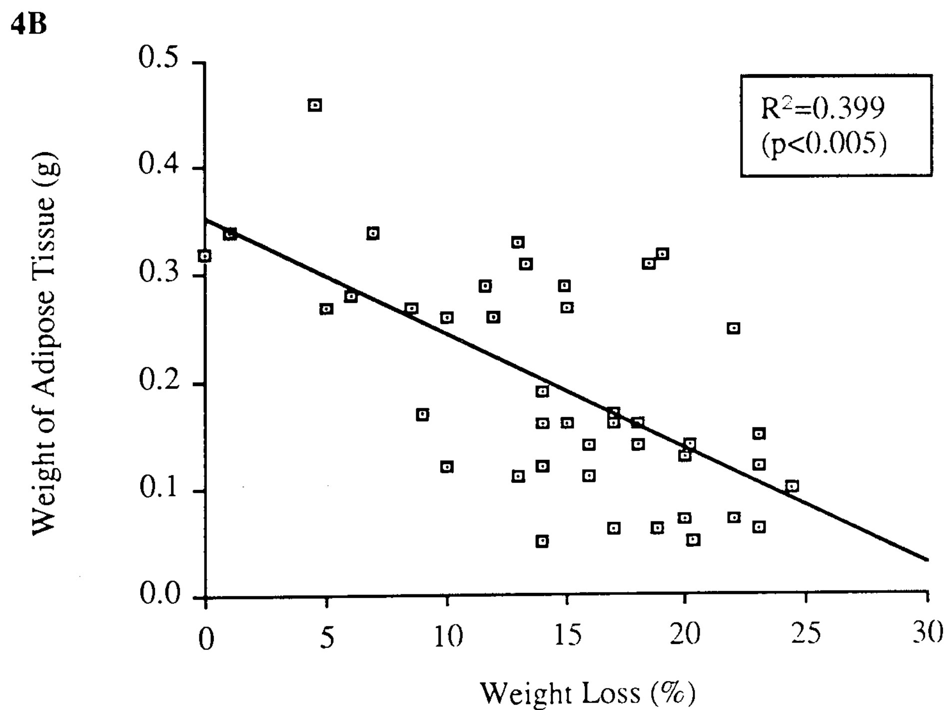
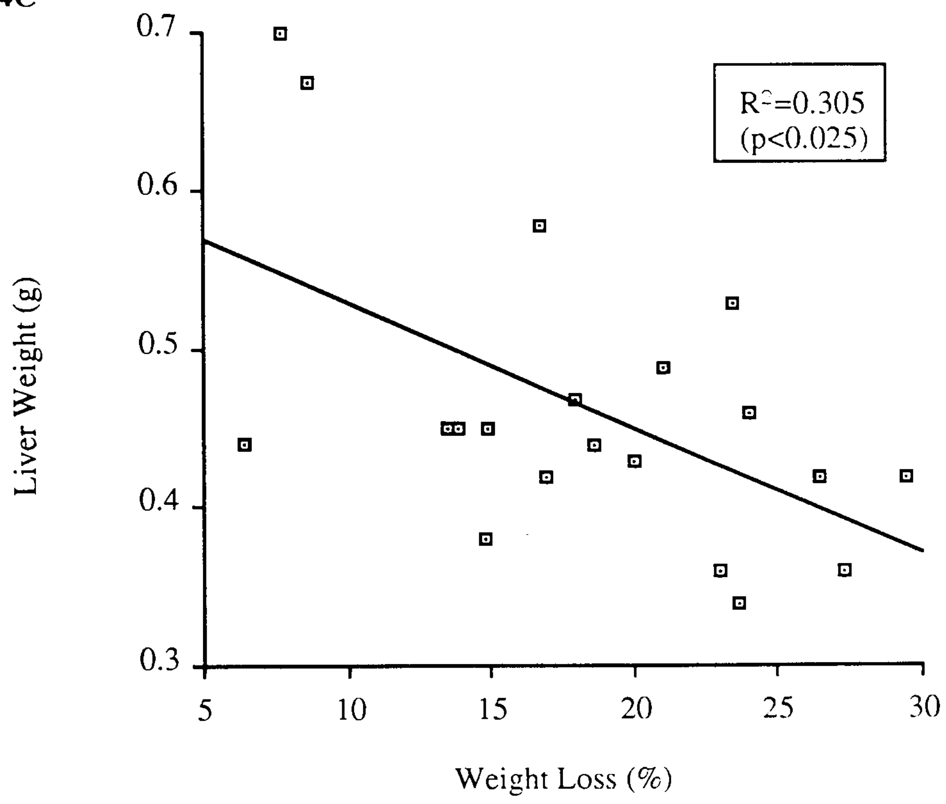


Figure 4. Relationship between weight loss (%) and tumour mass (A), weight of adipose tissue (B) and liver weight (C) of MAC16 tumour-bearing mice.
Each point represents results for one animal.
Results were analysed by linear regression followed by ANOVA.

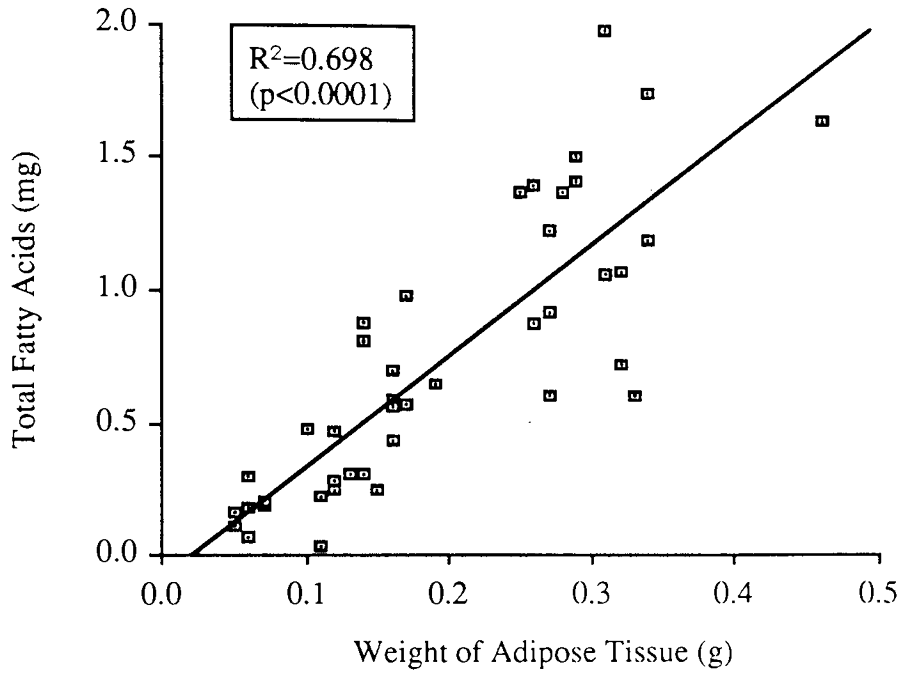
4C



The total fatty acid content (mg) of adipose tissue was found to correlate with both weight of adipose tissue and weight loss (Fig.5). Fatty acid composition of adipose tissue was found not to vary with weight loss (results not shown).

The specific activity of total liver fatty acids was found to increase with weight loss and to decrease with increasing liver mass (Figs. 6A and B). All fatty acids exhibited the trend to increase specific activity with weight loss, although only with palmitic acid (PA) and DHA did this relationship reach significance (Figs. 6C and D). All fatty acids detected in the liver (PA, SA, OA, LA, AA and DHA) showed an inverse correlation between specific concentration and liver mass (Figs. 6E - J). No correlation was found between liver weight and total mouse weight in control non tumour-bearing mice (weight range 17-22g) in contrast to the relationship found in cachectic animals. The specific concentration of individual liver fatty acids was not found to correlate with organ weight (results not shown), although total fatty acid concentration (mg/g) was found to increase with mouse weight (Fig.7). No correlation was found between liver total fatty acid content (mg) and organ weight, or mouse body weight/ weight loss in either non tumour-bearing or MAC16 tumour-bearing animals. The fatty acid composition of the liver of non tumour-bearing mice is shown in Table 2.

An increase in total plasma fatty acid concentration with weight loss was found to be due in part to an increase in circulatory concentration of arachidonic acid (AA) (Figs. 8A and B). A trend for a general increase in the plasma concentration of all fatty acids was noted, but only for AA did this increase reach significance. This increase in AA concentration occurred in conjunction with a decrease in the LA:AA ratio (Fig. 8C), although no change in plasma LA concentration was observed. The fatty acid composition of plasma of non tumour-bearing mice is shown in Table 2. In both the liver and plasma of MAC16 tumour-bearing mice with a weight loss of 5-10%, the concentration of fatty acids were found to be decreased when compared with non tumour-bearing controls (Table 2).



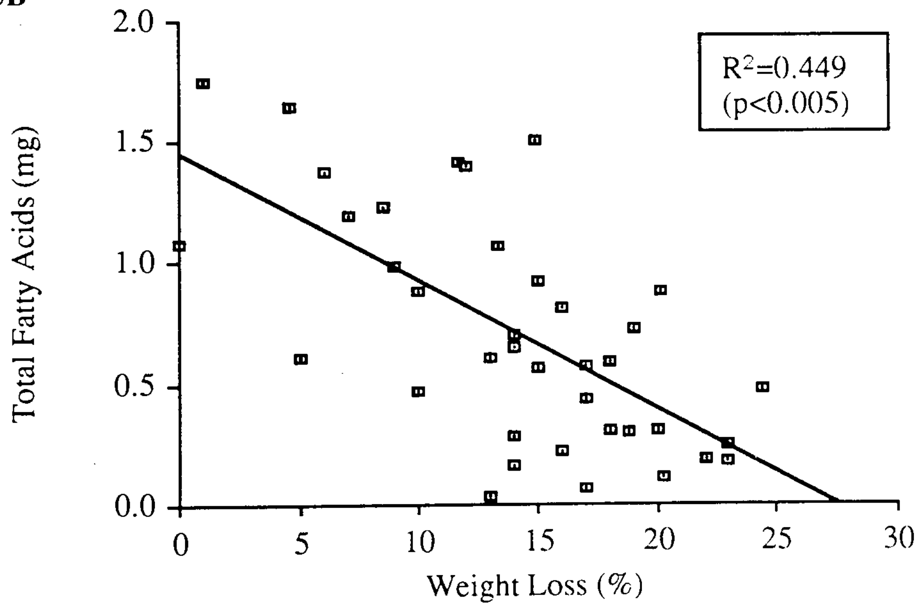
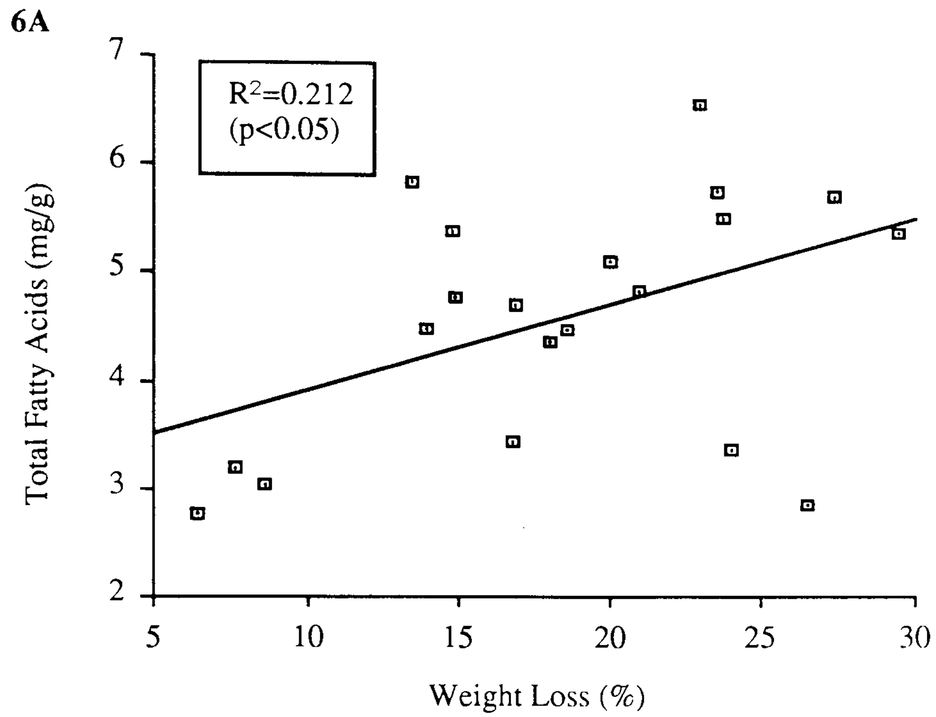


Figure 5. Relationship between total fatty acids (mg) of adipose tissue and weight of adipose tissue (A) and weight loss (B) in MAC16 tumour-bearing mice.

6A



6B

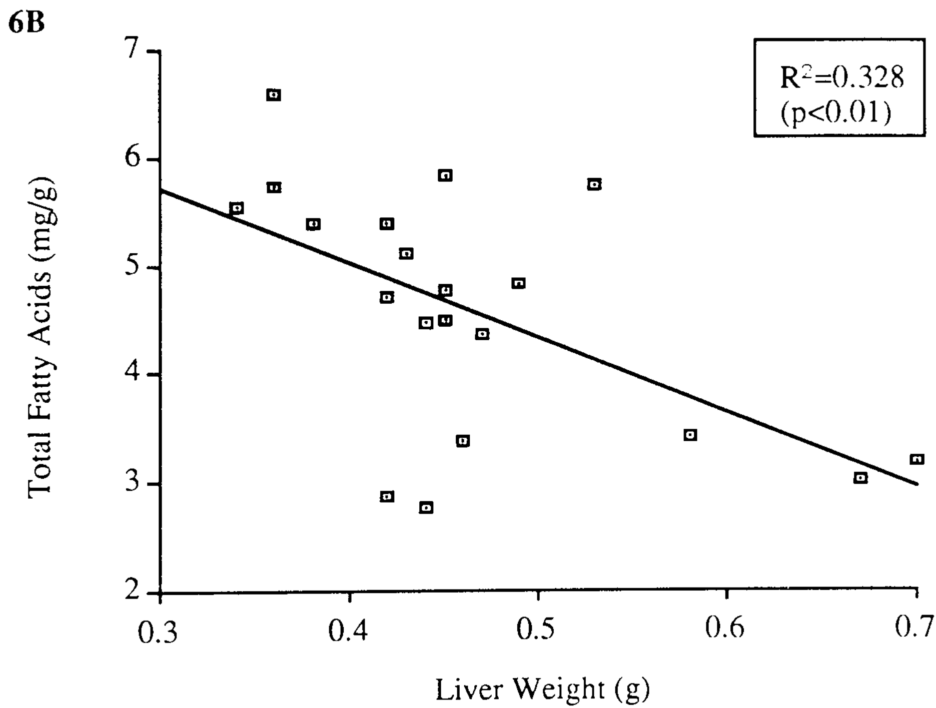
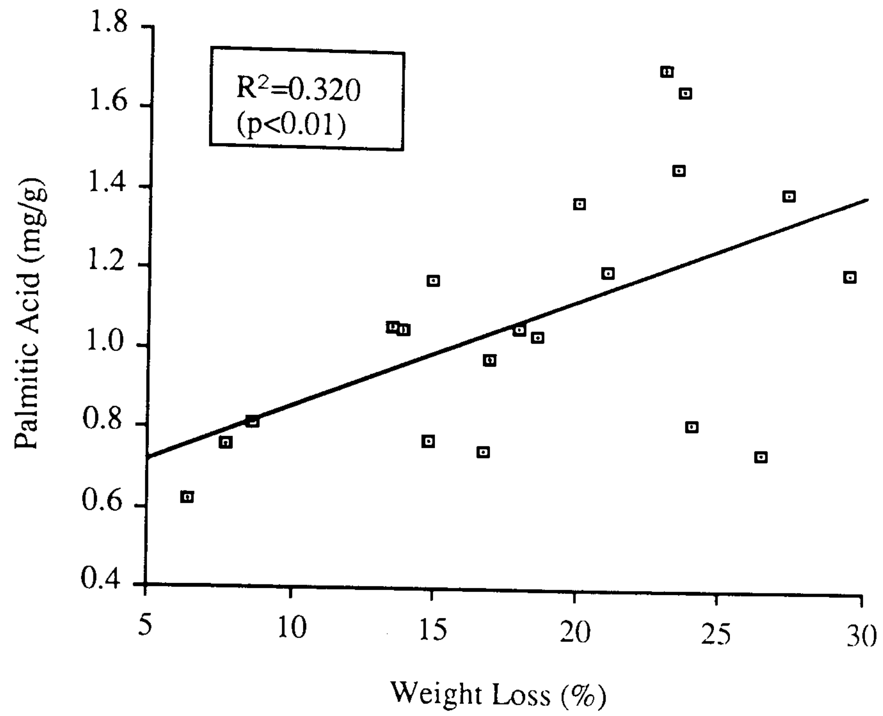
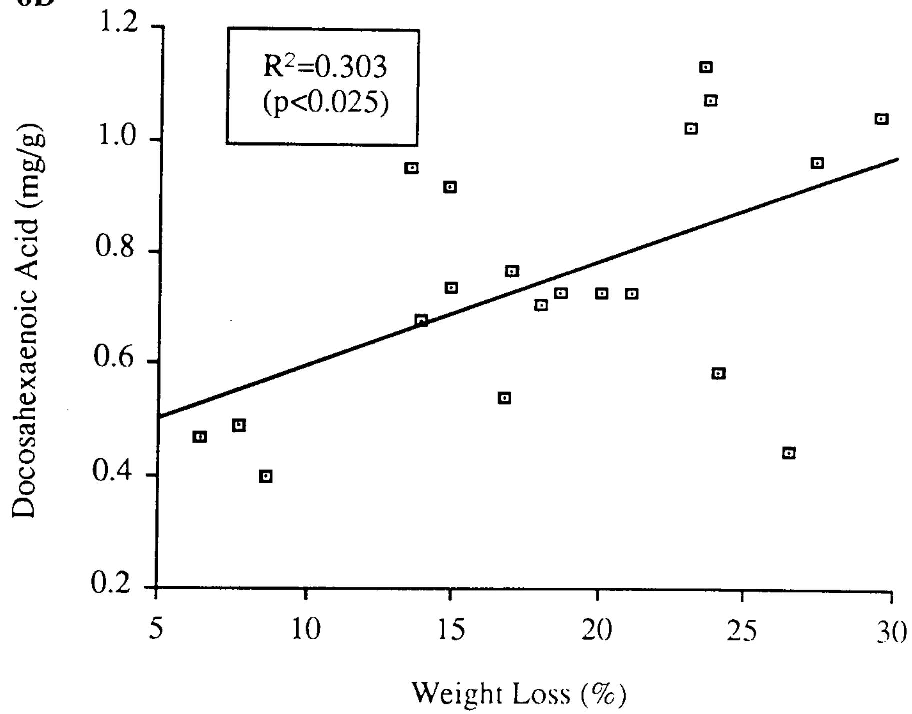
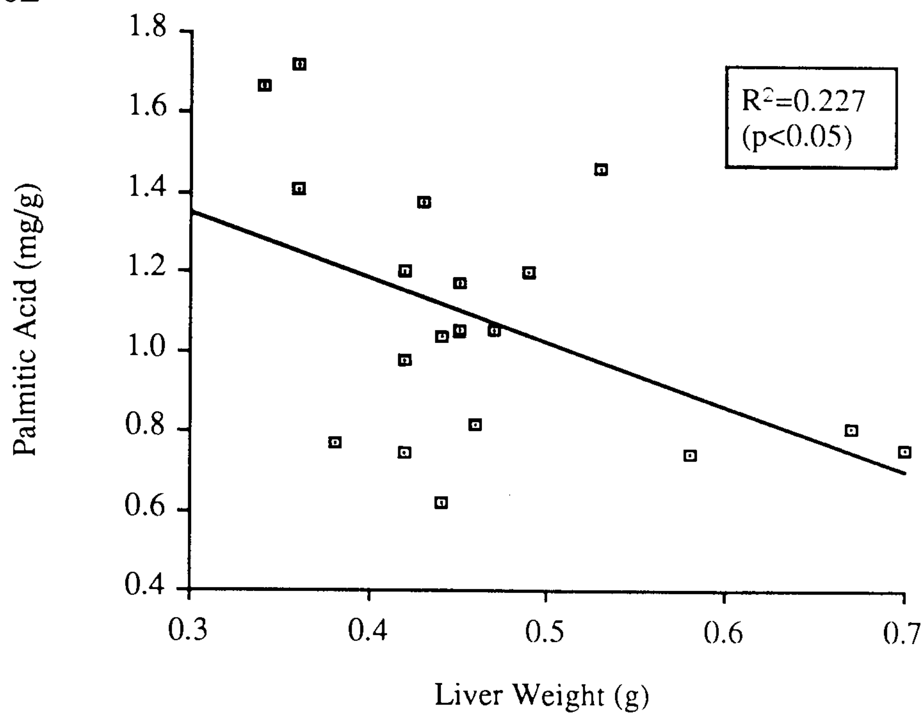


Figure 6. Relationship between total fatty acid concentration of the liver and weight loss (A), and liver weight (B); between weight loss and liver fatty acid specific concentrations of PA (C) and DHA (D); and between liver weight and liver fatty acid specific concentrations of : PA (E), SA (F), OA (G), LA (H), AA (I) and DHA (J).

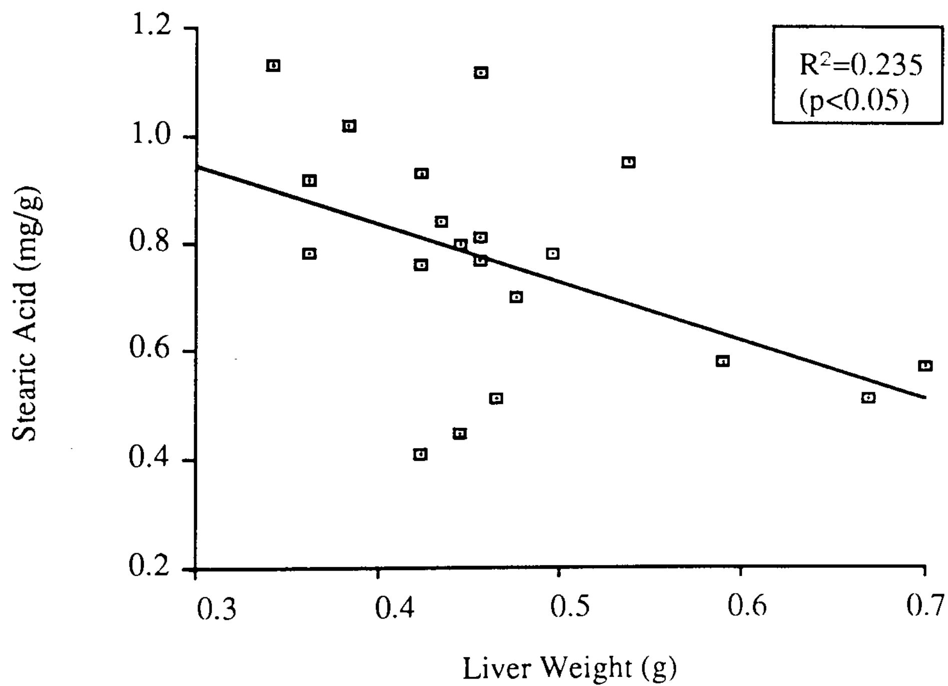

6C
R²=0.320
(p<0.01)
Palmitic Acid (mg/g)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
5
10
15
20
25
30
Weight Loss (%)


6D
R²=0.303
(p<0.025)
Docosahexaenoic Acid (mg/g)
1.2
1.0
0.8
0.6
0.4
0.2
5
10
15
20
25
30
Weight Loss (%)

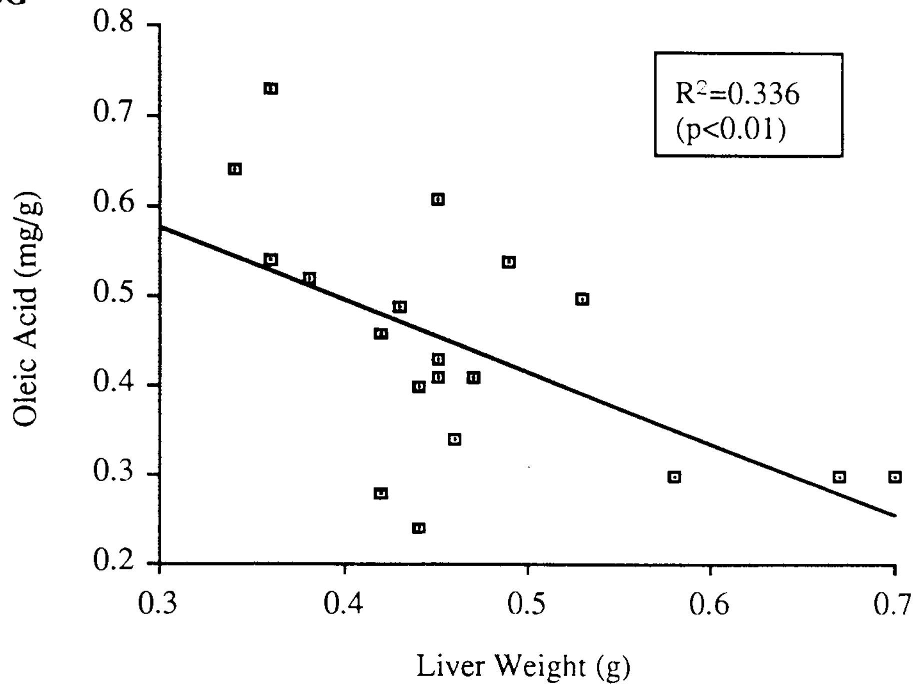
6E



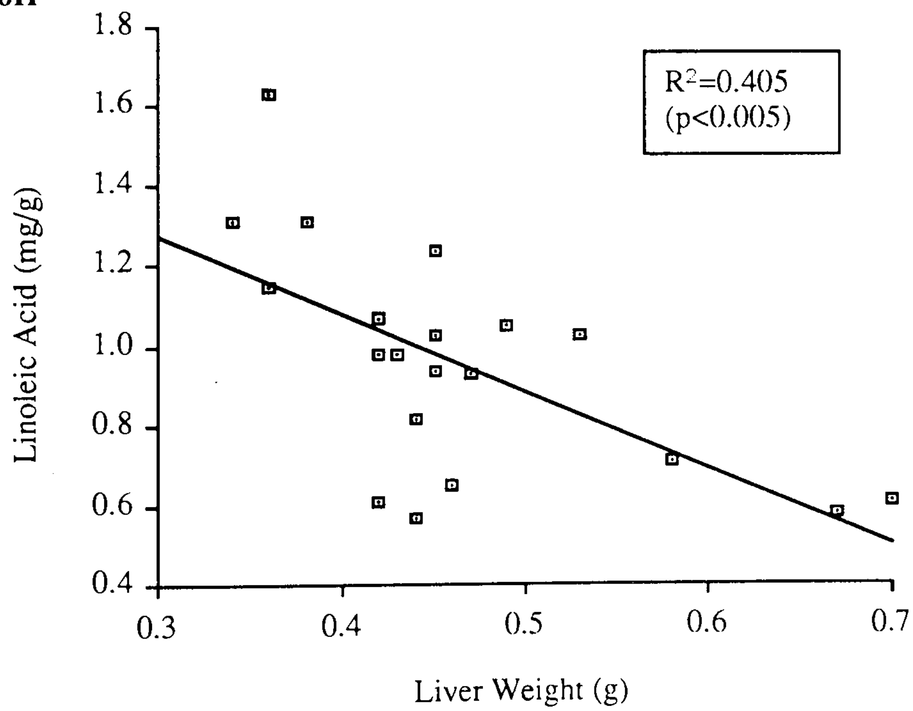
6F



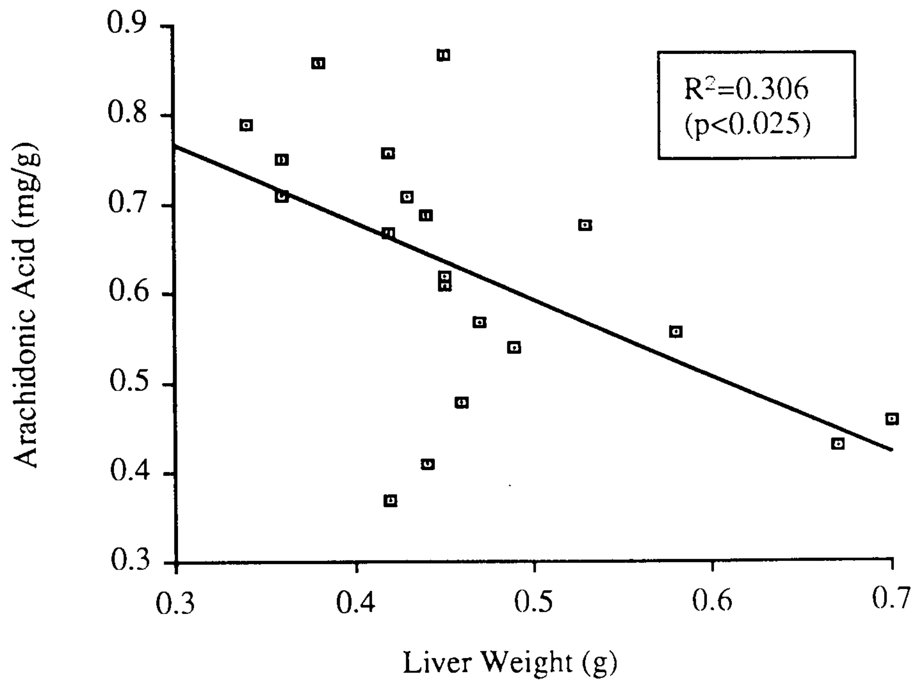
6G



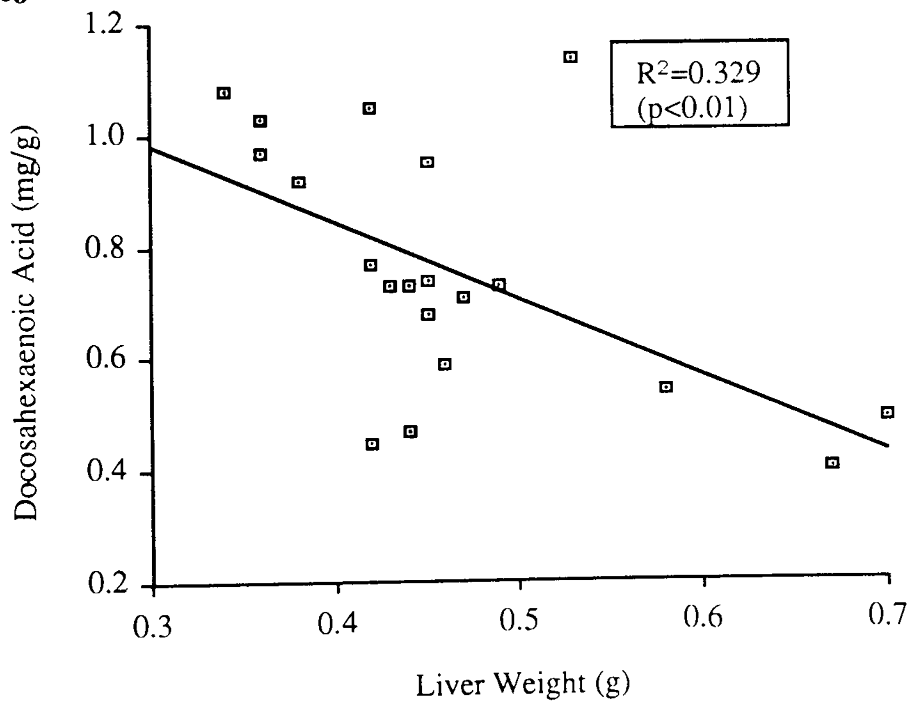
6H



6I



6J



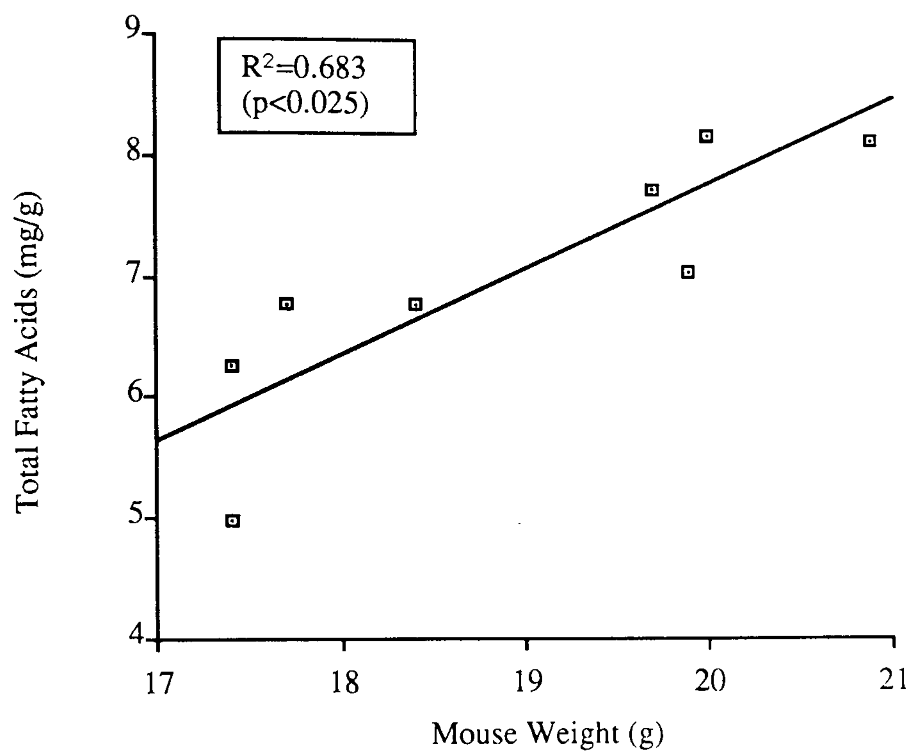
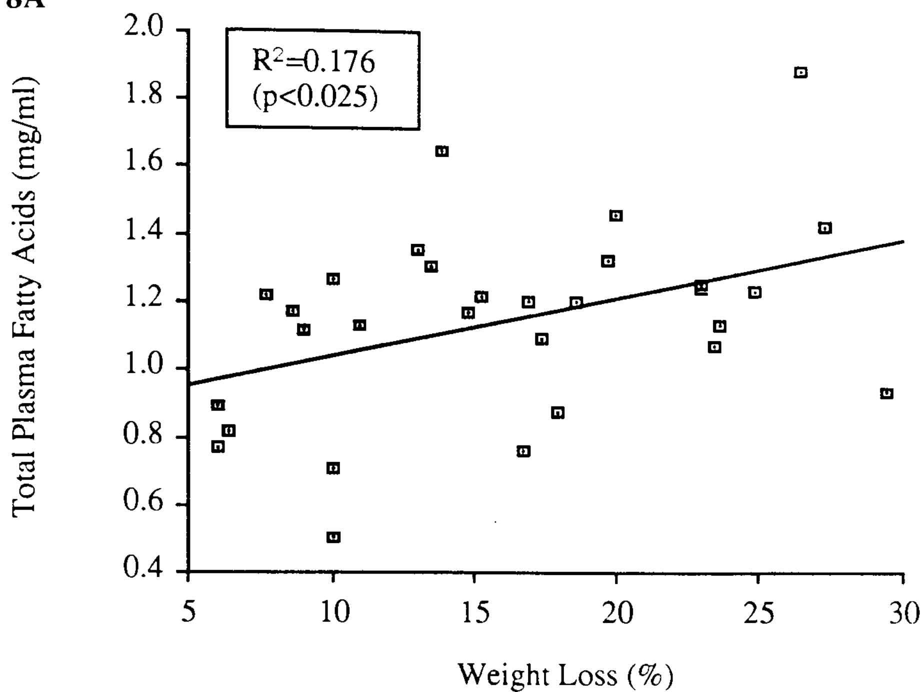


Figure 7. Relationship between total liver fatty acid specific concentration and body weight of non tumour-bearing female NMRI mice.

Fatty Acid	NTB Liver (mg/g)	NTB Plasma (mg/ml)
16:0	1.68 ± 0.12	0.38 ± 0.02
18:0	1.35 ± 0.10	0.17 ± 0.008
18:1 (n-9)	0.85 ± 0.06	0.29 ± 0.02
18:2 (n-6)	1.34 ± 0.09	0.52 ± 0.03
20:4 (n-6)	0.98 ± 0.07	0.12 ± 0.02
20:5 (n-3)	0.09 ± 0.01	0.01 ± 0.003
22:6 (n-3)	1.06 ± 0.09	0.08 ± 0.006
Total	7.33 ± 0.61	1.57 ± 0.06

Table 2. Fatty acid composition of the plasma and livers of non tumour-bearing (NTB) female NMRI mice.
Results are expressed as mean ± SEM.

8A



8B

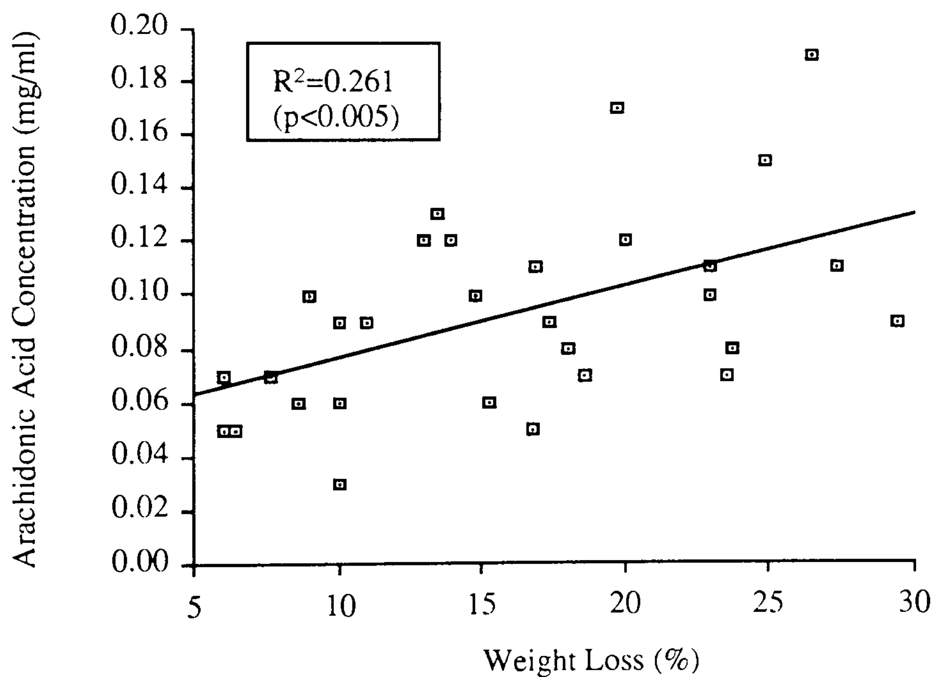
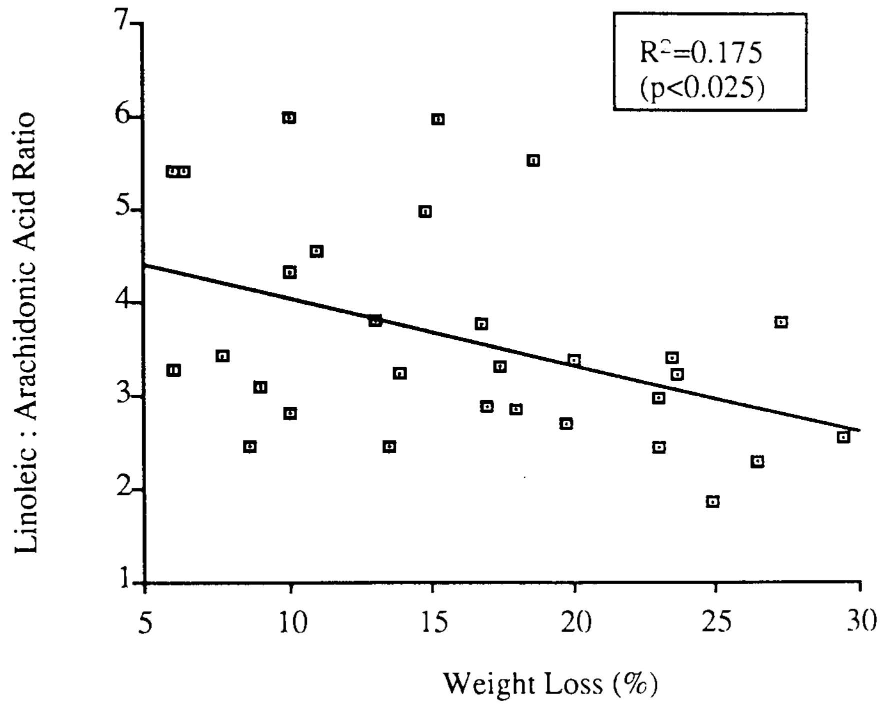


Figure 8. Relationship between weight loss and total plasma fatty acid concentration (A), plasma AA concentration (B), and plasma LA:AA ratio (C).

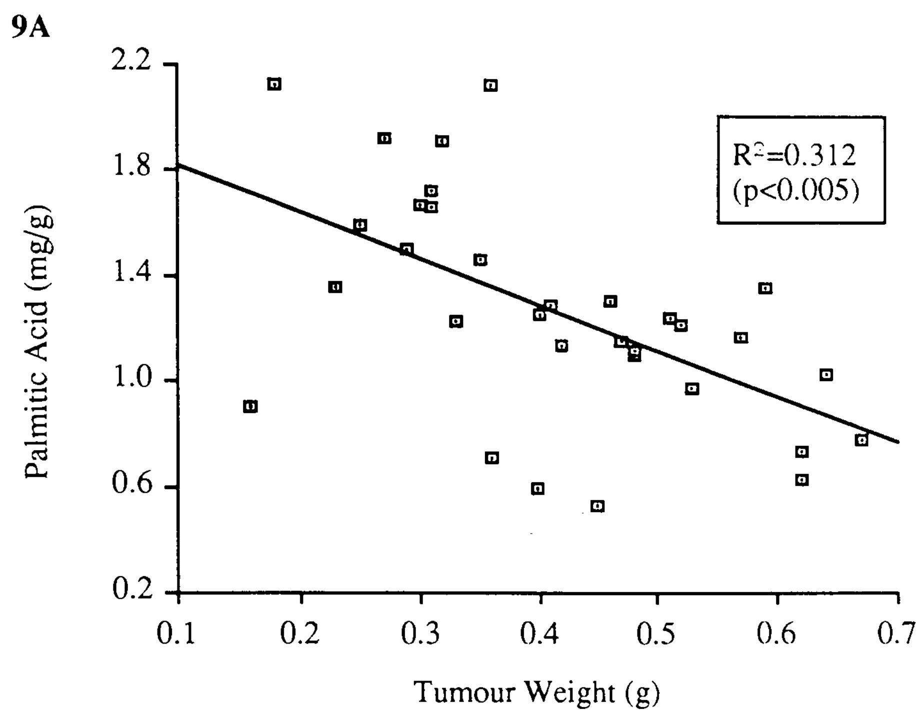
8C



The specific activity of tumour fatty acids was found to decrease with increasing tumour mass (Fig.9A-F) with the exception of AA and DHA, the concentrations of which showed no simple relationship with tumour growth. Absolute amounts of individual fatty acids were however found to increase with tumour mass (Fig.10A-H), and for stearic acid (SA), LA, AA, DHA, and the total fatty acid content of tumour, the increase also correlated with weight loss ($p<0.01$, 0.005, 0.005, 0.05 and 0.02 respectively; results not shown). The ratio of SA to oleic acid (OA) was also found to increase in the tumour with both tumour mass and weight loss (Fig. 11A and B).

Results obtained from serum of cachectic human cancer patients are shown in Figure 12. A peak in AA concentration was found to occur between 10% and 15% weight loss, although this was not found to show statistical significance (Fig.12A). In contrast to the results obtained in the tumour of MAC16 tumour-bearing mice, in human serum the SA:OA ratio was found to decrease with increasing weight loss (Fig. 12B).

9A



9B

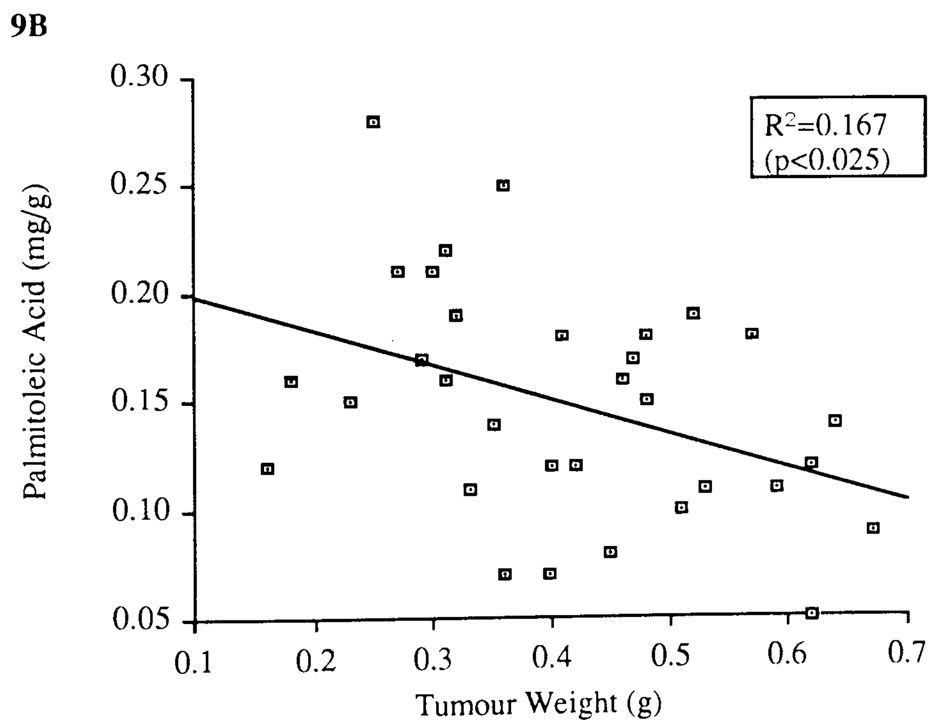
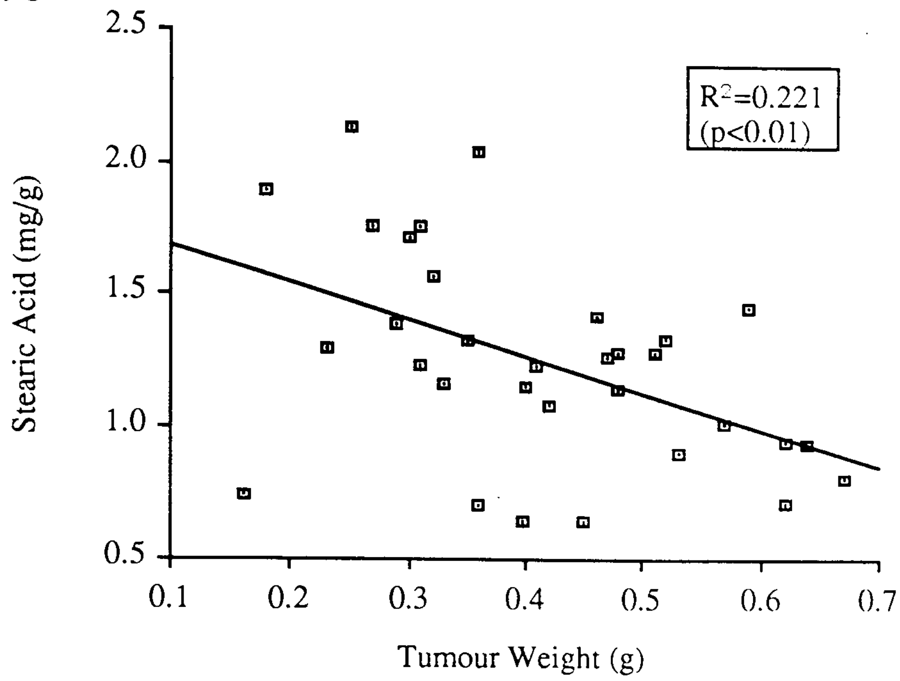
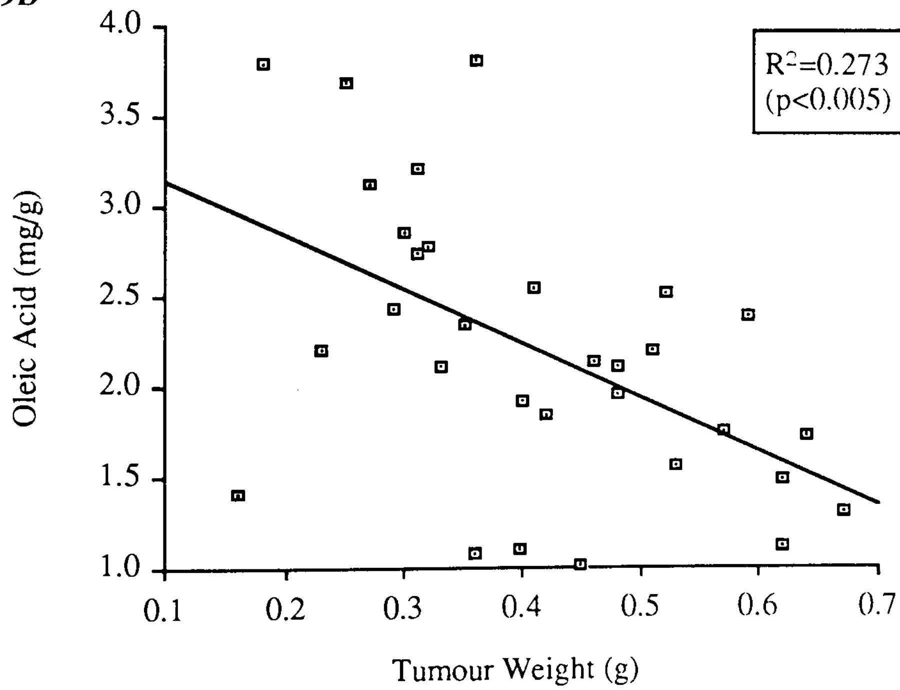


Figure 9. Relationship between tumour weight and the tumour specific concentration of PA (A), POA (B), SA (C), OA (D), LA (E), and total fatty acids (F).

9C



9D



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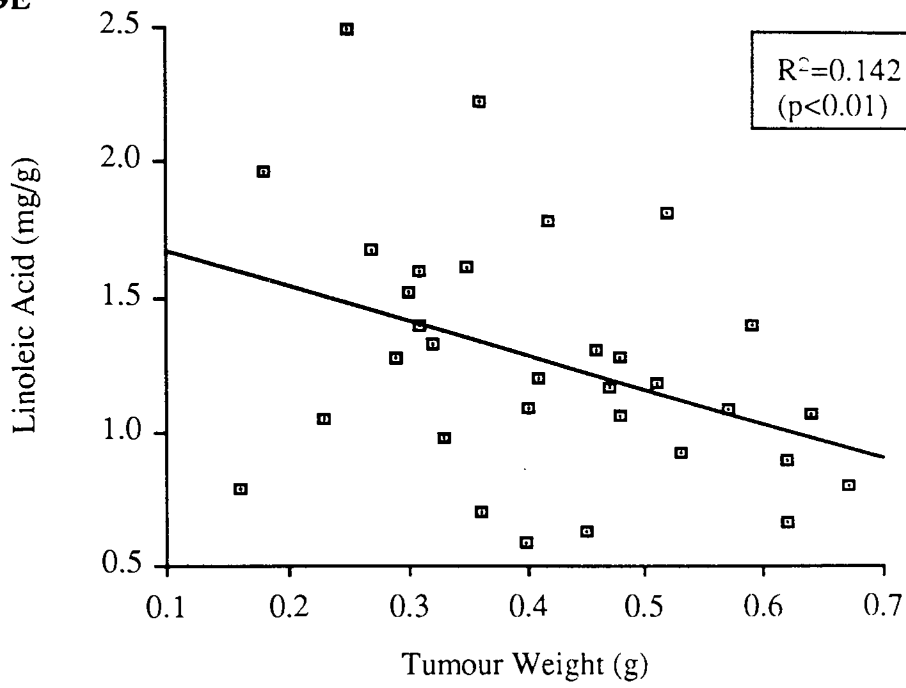

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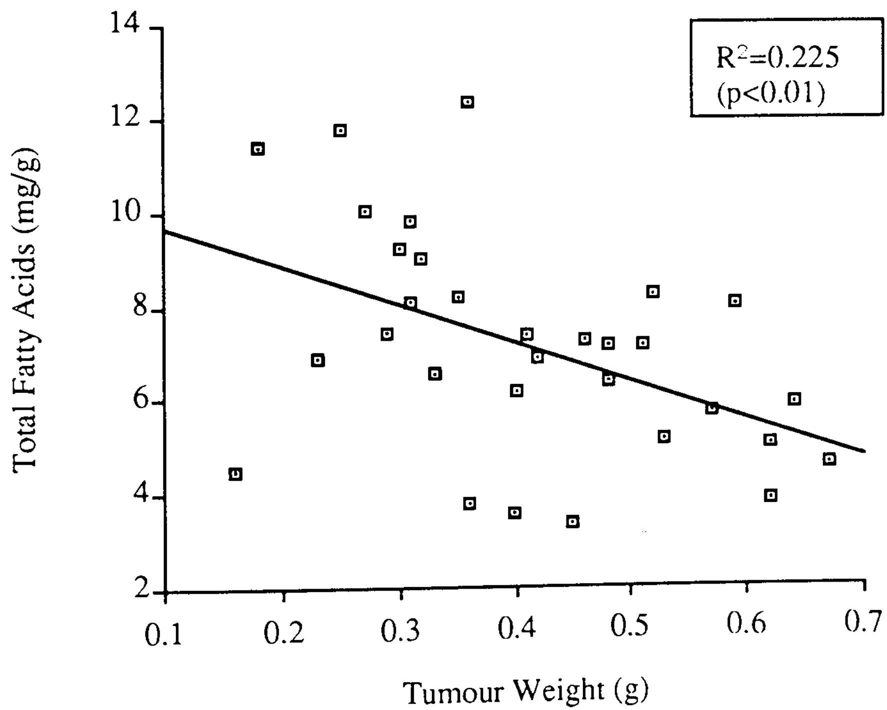
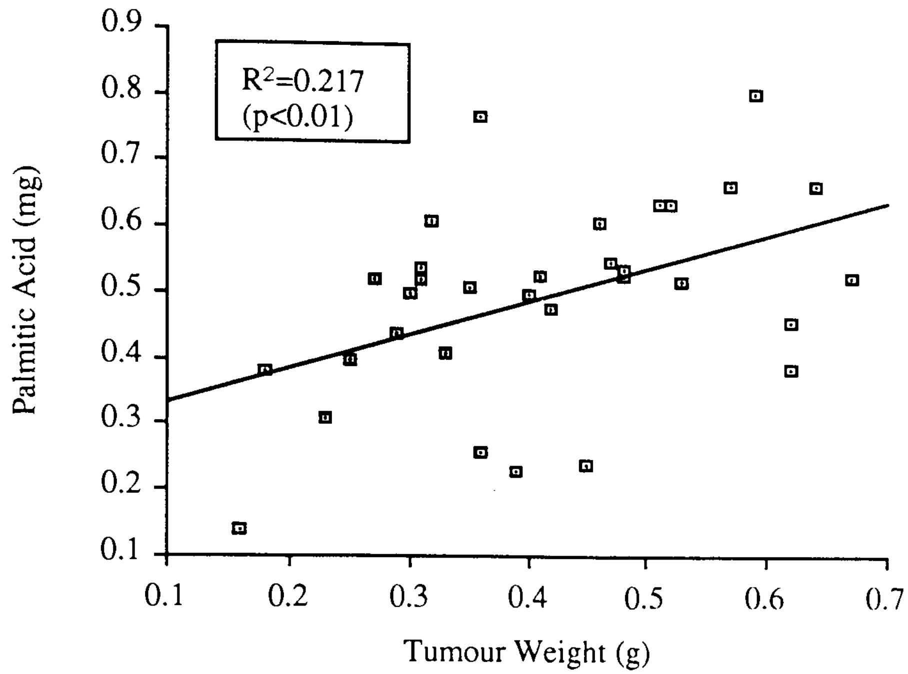


10A



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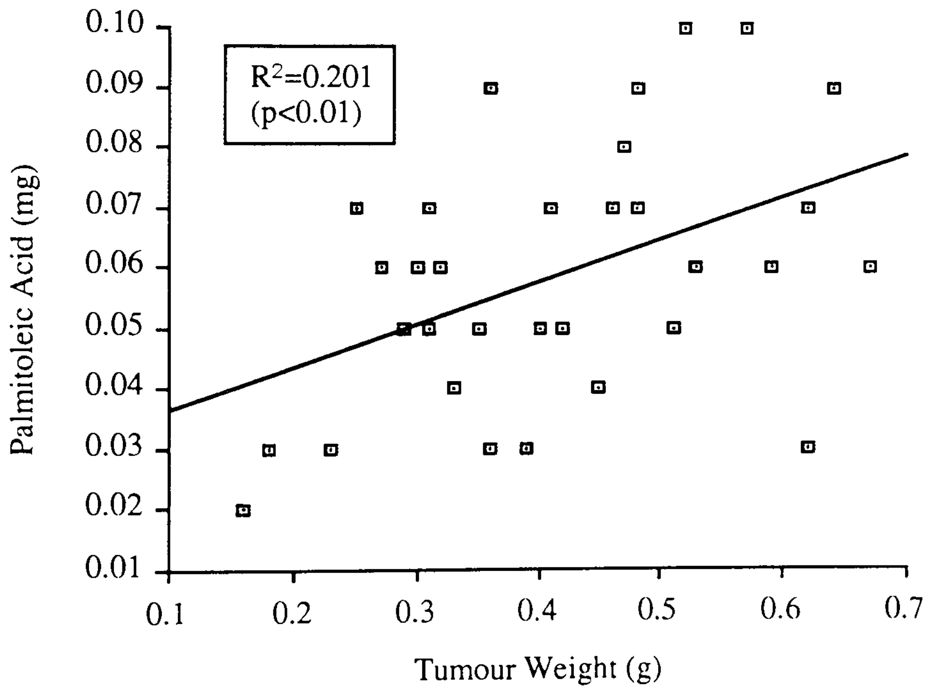
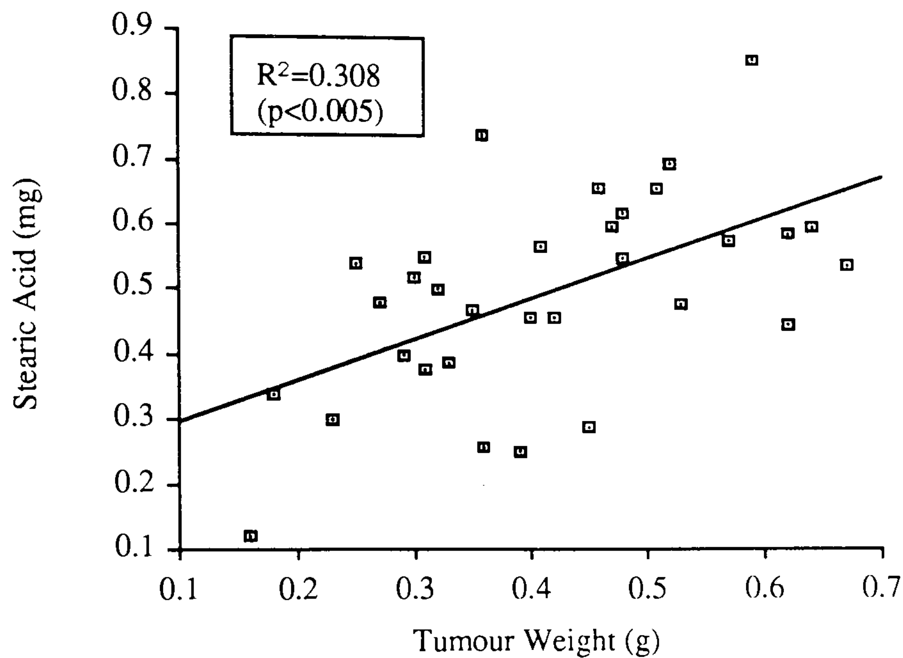


Figure 10. Relationship between tumour weight and tumour content (mg) of PA(A), POA (B), SA (C), OA (D), LA (E), AA (F), DHA (G) and total fatty acids (H).

10C



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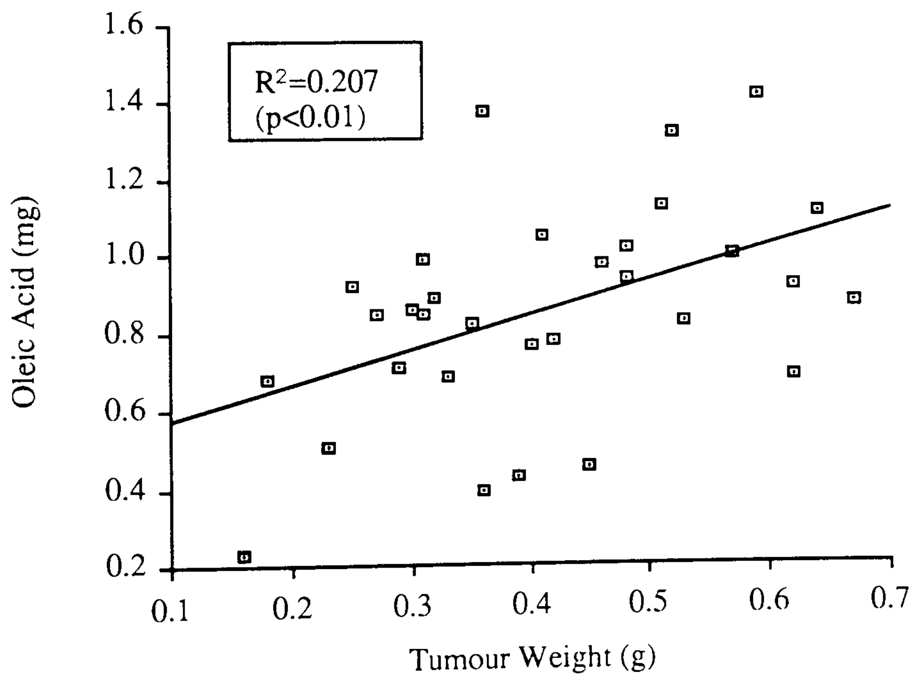

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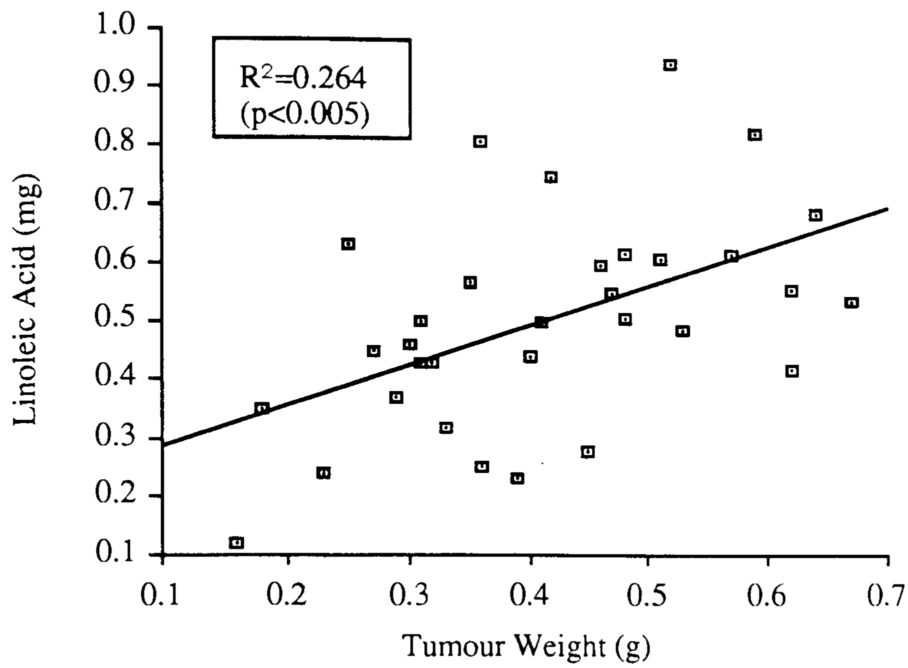

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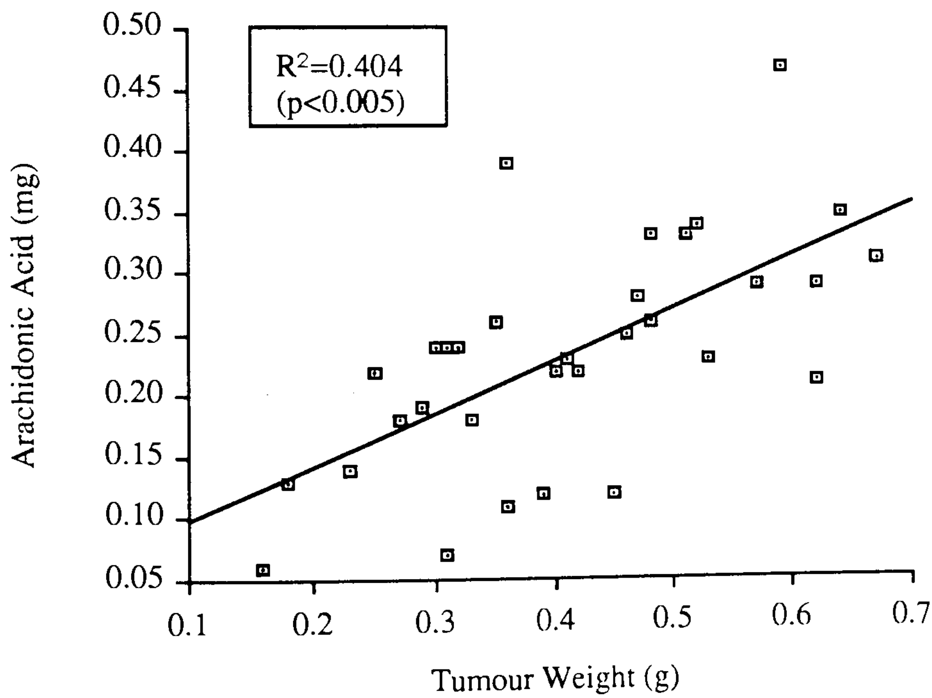

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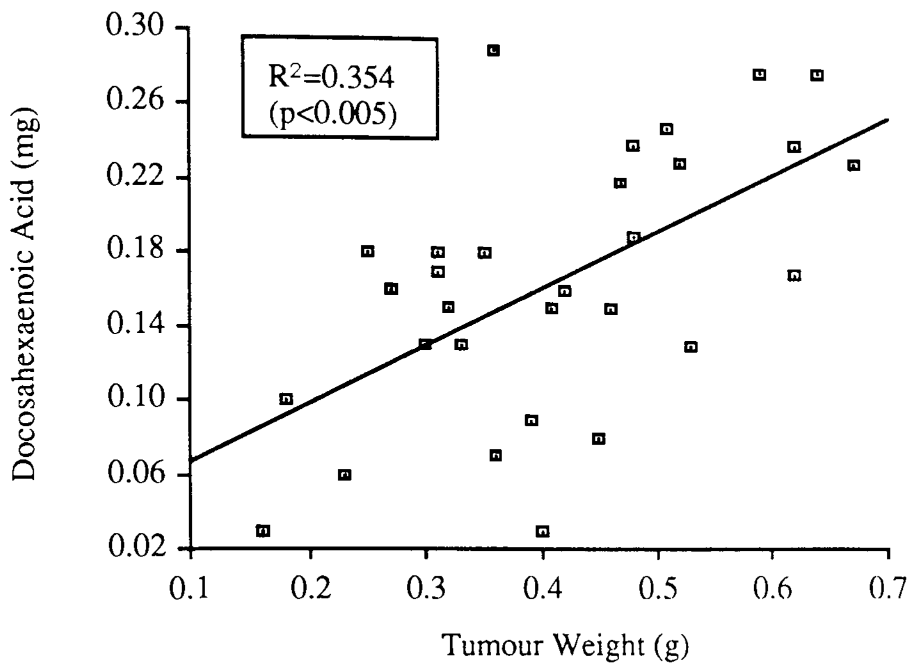
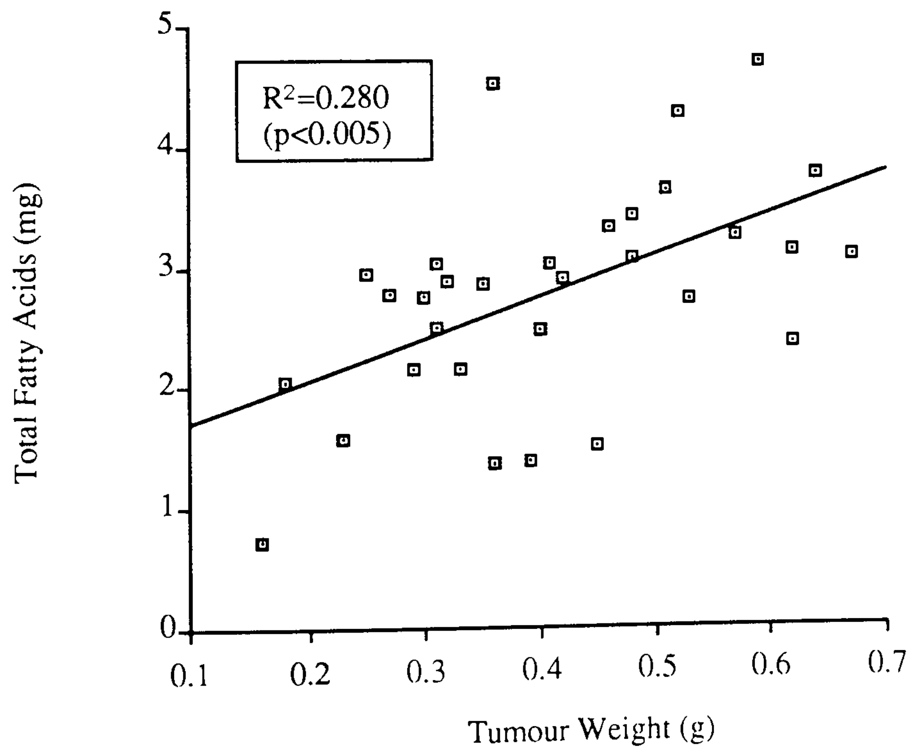
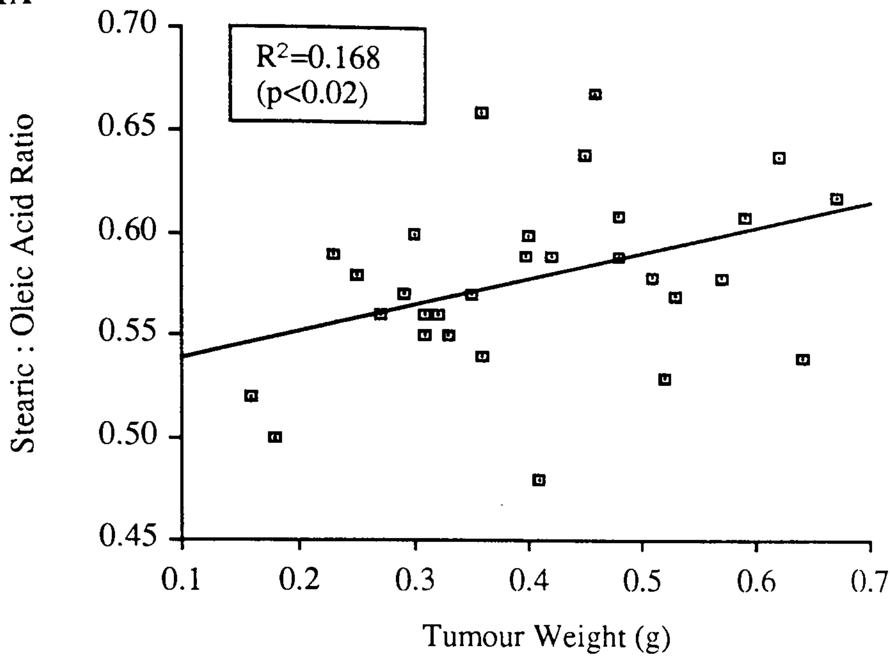


10H



11A



11B

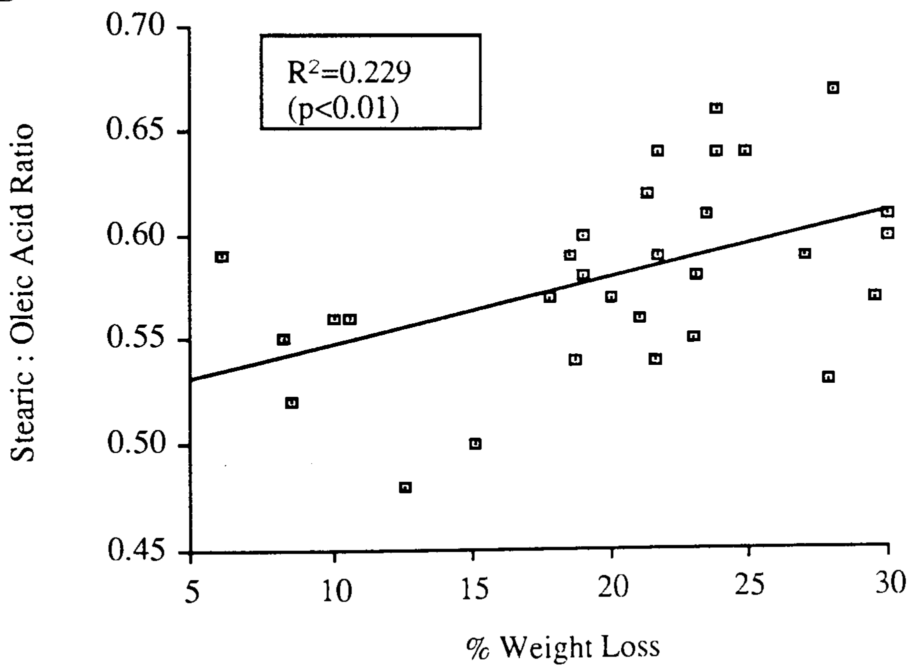
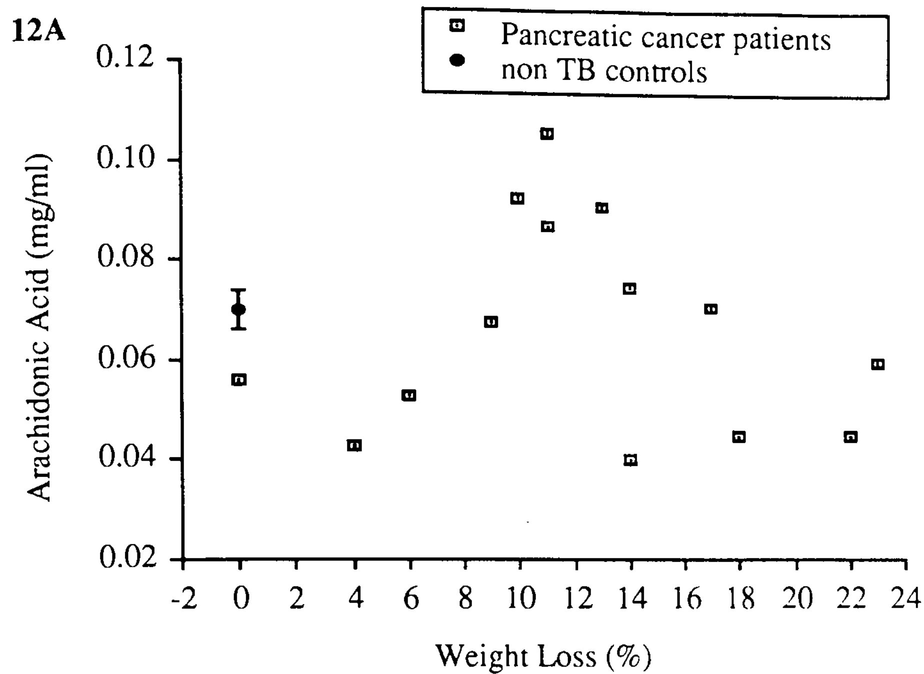


Figure 11. Relationship between the ratio of stearic to oleic acid in tumours and tumour weight (A), and weight loss (B).



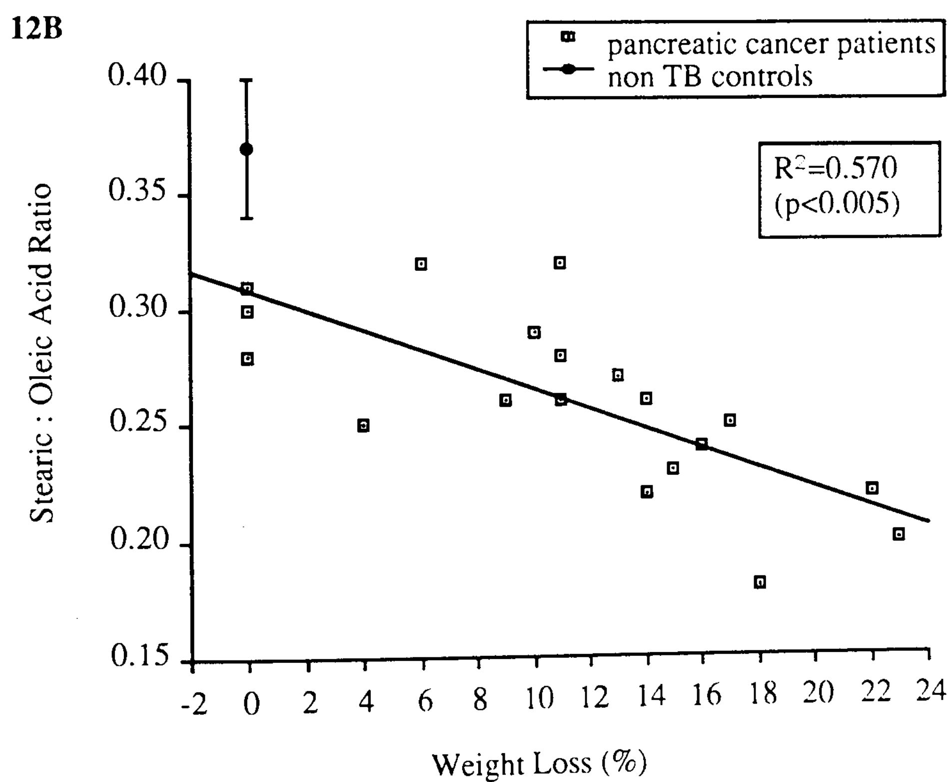


Figure 12. Relationship between weight loss of pancreatic cancer patients and plasma AA concentration (A), and the plasma stearic to oleic acid ratio (B).

4.1.3 Discussion.

Extensive weight loss with a small tumour burden is characteristic of the MAC16 model of cachexia. In this study weight loss was found to increase linearly up to 30% in animals with tumour burdens between as little as 1% and 6% of the host body weight. The decrease in adipose mass and fatty acid content of the tissue in response to cachexia, without changes in the specific fatty acid composition of the tissue, confirms that lipid stores are mobilised in this model, but indicates that the process is not selective for any particular fatty acid.

In this study, liver wet weight was also found to decrease with increasing weight loss; this is in contrast to previous reports of liver enlargement in cancer cachexia, and an increase in the dry liver weight : total body weight ratio (Theologides, 1972; Shamberger, 1984). The decrease in liver mass reported here is not thought to be due to a decrease in water content, as previous studies with the MAC16 model have shown liver weight as a percentage of total body weight not to vary from control non tumour-bearing animals (Mulligan and Tisdale, 1991b). Also total body water content as a percentage of body mass has been found not to vary with the progression of cachexia (Smith, 1992) indicating that changes in organ weights are not due simply to a loss of water.

The inverse correlation between liver fatty acid concentration and its mass suggests that as the liver decreases in size in response to tumour growth and cachexia, it has an increased capacity to concentrate fatty acids released into the circulation from adipose stores; as with adipose tissue, the changes in fatty acid concentration were non-specific. This suggestion is supported by the finding that no correlation occurs between liver weight and mouse body weight, or between liver fatty acid concentration and organ weight in non tumour-bearing animals, and that in contrast to the tumour-bearing state, the specific activity of liver fatty acids increases with the size of the non tumour-bearing animal, indicating that the changes observed in the livers of tumour-bearing mice occur as a direct result of tumour growth and cachexia.

Previous studies with this model have shown the liver to be the major organ of palmitate consumption, its oxidation to CO_2 being twice the control levels (Mulligan *et al.*, 1992b). This suggests that not only may fatty acids be required by the tumour for growth processes, but that fatty acids released by adipose tissue during cachexia may provide an energy source for other tissues.

The release of fatty acids from lipid stores is also reflected by the increase in plasma total fatty acid concentration with weight loss. The levels of both plasma and liver fatty acid concentration of MAC16 tumour-bearing mice with 5-10% weight loss were found to be lower than levels of control non tumour-bearing mice. This reflects the lag period of tumour growth which occurs prior to the onset of weight loss, and during which time the growing tumour may sequester fatty acids from the plasma and liver of the host, causing a drop in fatty acid concentration. Production of lipolytic factors by the tumour causing mobilisation of fat stores and release of fatty acids into the circulation may then account for the rise in fatty acid concentration seen in plasma and liver in response to weight loss. Plasma unsaturated fatty acids have also been reported to be raised in human cancer patients (Mueller and Watkin, 1961).

The decrease in the murine plasma LA:AA ratio observed in this study was attributed to an increase in the circulating AA concentration in response to weight loss; this was supported by results obtained with human cancer patient serum in which a peak in AA appears to occur at around 10% to 15% weight loss. This may indicate a role for circulating AA in the development of cachexia. Mosconi *et al.* (1989) have reported a decrease in the LA:AA ratio of the plasma cholesterol ester fraction in malnourished cancer patients, although in contrast to results presented here, a decrease in LA as a percentage of total plasma phospholipids was implicated in the weight loss experienced by the patients. Chaudry *et al.* (1981) have also documented changes in the fatty acid composition of the phospholipid fraction of plasma in cancer patients, consisting of an increased oleic acid concentration compared to controls. Unfortunately, attempts to fractionate plasma obtained from cachectic tumour-bearing female NMRI mice, by the method of Brenner and Nervi (1965) were unsuccessful. This was attributed to the small volume of plasma

obtainable from these mice, and lack of fatty acid methyl ester detection sensitivity due to the use of a packed column gas chromatogram.

Studies reported here on the fatty acid composition of tumours indicate that while the fatty acid content of the tumour increases as it grows, the specific activity of the fatty acids in the tumour actually decreases.

Recently, a great deal of attention has been focussed on changes in the SA:OA ratio of neoplastic tissue compared with control tissue. The ratio has been found to be decreased in the erythrocyte membranes of patients with malignant liver conditions (Habib *et al.*, 1987a; 1987b; Wood *et al.*,1985a) and leukaemia compared with healthy controls (Apostolov *et al.*, 1985), and also in neoplastic liver tissue when compared with unaffected areas of tissue (Wood *et al.*, 1985b; Habib *et al.*, 1985a). The SA:OA ratio has also been found to decrease in parallel with the appearance of rodent tumours *in vivo* (Habib *et al.*, 1987c). It has been suggested that this increase in desaturation may increase the membrane fluidity of tumour cells (Apostolov *et al.*, 1985; Habib *et al.*, 1987d), possibly conferring an advantage on tumour cells over less fluid non cancerous cells. Mosconi *et al.* (1989) have reported a decrease in the SA:OA ratio in the plasma of cachectic cancer patients, a finding which is supported by the study of cachectic pancreatic cancer patients reported here. Other workers however, have found no change in the erythrocyte membrane of patients with colorectal cancer (Neoptolemos and Thomas, 1990), and have found an increase in the SA:OA in cancerous colorectal tissue compared with normal mucosa (Neoptolemos *et al.*, 1991). This report by Neoptolemos *et al.* (1991) agrees with the increase in SA:OA ratio in response to both tumour growth and cachexia observed in this study with the MAC16, a murine colon adenocarcinoma. The discrepancy in results reported, may indicate a different relationship between the SA:OA ratio in colon-related cancer than in other cancers such as tumours of the liver and pancreas.

In summary, adipose tissue was found to be depleted during cachexia in this model in a non-specific manner, leading to an increase in plasma fatty acid levels with weight loss. An increased capacity for uptake of fatty acids by the liver was also proposed as a result of the finding of an increase in fatty acid specific activity

with decreasing liver size in response to weight loss. Tumour fatty acid content increased with growth, although specific activity decreased. Most changes observed in these organs were found to be non-specific for individual fatty acids with the exception of an increase in plasma concentration of AA in MAC16 tumour-bearing mice, and a peak in AA in the serum of pancreatic cancer patients with between 10 and 15% weight loss. An increase in the tumour SA:OA ratio was observed with both increasing tumour size and weight loss in MAC16 tumour-bearing mice, which was found to be in contrast to the decrease in the ratio obtained in the serum of the human patients.

4.2 Effect of Treatment with PUFAs on the Tumour Growth and Weight Loss of MAC16 Tumour-bearing Mice, and on the Fatty Acid Composition of Tissues.

4.2.1 Introduction.

Evidence for the involvement of dietary fat in tumourigenesis has accumulated over recent years, as discussed in section 1.4. Of particular concern is the n-6 PUFA LA, a major constituent of many low fat margerines promoted as a healthy alternative to butter (France and Brown, 1991). LA in the form of a 10 or 20% corn oil diet has been found to promote tumour development in rodent models (Abraham and Hillyard, 1983; Jurkowski and Cave, 1985), and the latent interval of tumour appearance has been found to be decreased, and subsequent growth rate of implanted breast cancer cells and metastasis increased in nude mice fed a LA rich diet (Rose *et al.*, 1991; Rose and Connolly, 1992).

Perfusion of tumours *in situ* with hyperlipidaemic blood has been found to increase [^{3}H]-thymidine incorporation into tumours (Sauer and Dauchy, 1988). This response was found to be due to the presence of LA or AA, and blood containing palmitic, stearic or oleic acids was found to be inactive. It was concluded from this study, that LA and AA are rate limiting for thymidine incorporation, suggesting a specific requirement of the tumour for one or both of these fatty acids.

In contrast, the n-3 fatty acids EPA and DHA in the form of fish oil have been found to have anti-tumour activity in a range of rodent models (Karmali *et al.*, 1984; 1987; Jurkowski and Cave, 1985; Kort *et al.*, 1987; Pritchard *et al.*, 1989; Reddy and Maryama, 1986; Gabor and Abraham, 1986; Sakaguchi *et al.*, 1990). Fish oil has also been found to possess anti-cachectic activity which has recently been determined to be specific to EPA (Tisdale and Beck, 1991; Beck *et al.*, 1991).

Preliminary studies by Beck and Tisdale with EPA in the MAC16 model of cachexia found that a preparation of a minimum of 80% pure EPA was required for

anti-tumour activity. When pure EPA was diluted to 57% with other fatty acids (DHA 6.7%, palmitoleic acid (POA) 13.5%, oleic acid (OA) 9.3%, stearic acid (SA) 4.4%, palmitic acid (PA) 2.1%) the anti-tumour activity but not the anti-cachectic activity was lost (Hudson *et al.*, 1993 - appendix 2). This suggests that the anti-tumour and anti-cachectic activity of EPA are exerted by different mechanisms, and that EPA may act by blocking the uptake by the tumour of a specific fatty acid which was present in the 57% preparation.

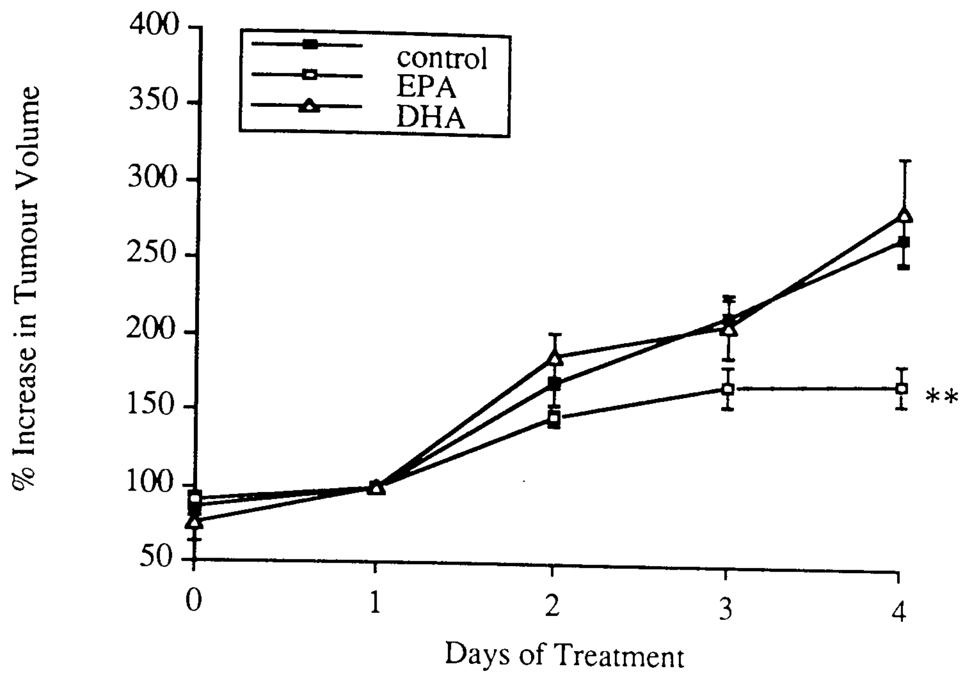
This study tests this hypothesis by administering EPA in combination with either LA (free acid or triglyceride) or OA, and by monitoring fatty acid composition of the tumour after the various PUFA treatments.

4.2.2 Results.

Results presented in Fig.13 confirm that DHA (2.25g/kg) does not possess either anti-tumour or anti-cachectic properties against the MAC16 model, and that therefore the activity of fish oil is due to the presence of EPA.

Figs.14 and 15A and B show that in combination with the PUFAs OA (2.0g/kg) and LA (1.9g/kg) the anti-tumour activity of orally administered EPA (2.0g/kg, 2.25g/kg) was reversed. The anti-cachectic activity of EPA however was not affected by either of the PUFAs. The stimulatory effect of LA on tumour growth (Fig.15A) was found to be variable, depending on the rate of tumour growth; growth of MAC16 tumours with a comparatively short doubling time (less than 48 hours (Fig.15C) compared with approximately 96 hours (Fig.15A)) was found not to be stimulated above the rate of growth occurring in control animals dosed with water (Fig.15C). Administration of corn oil (CO) (5.0g/kg) containing triglycerides of LA 60.3%, OA 28.2% and PA 11.5% (by gas chromatography) was found to have no effect on tumour growth or weight loss of MAC16 tumour-bearing animals, and did not reverse the activity of EPA (Fig.16).

13A



13B

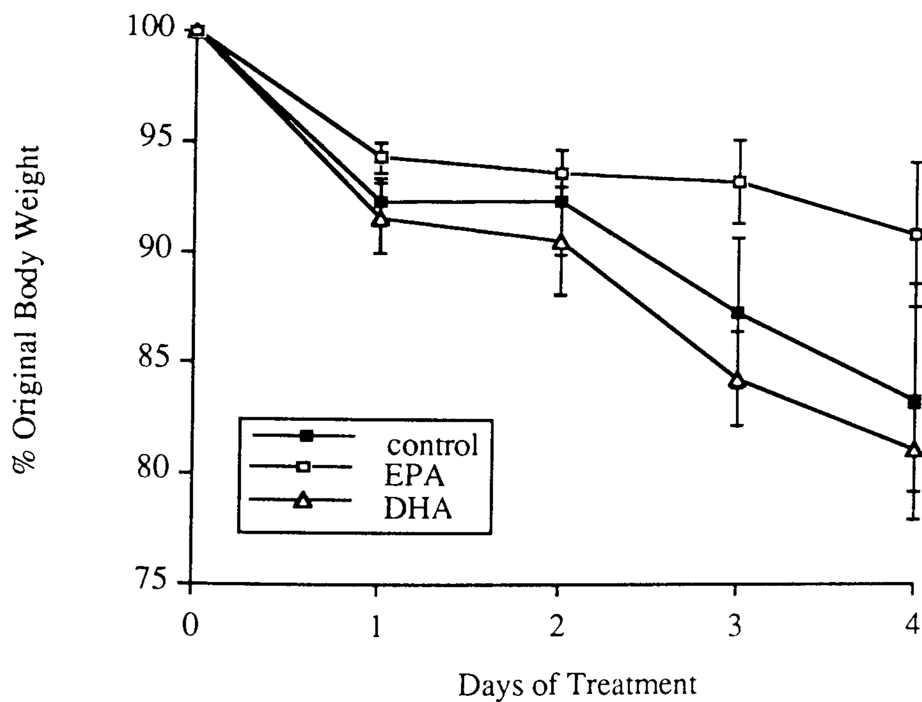
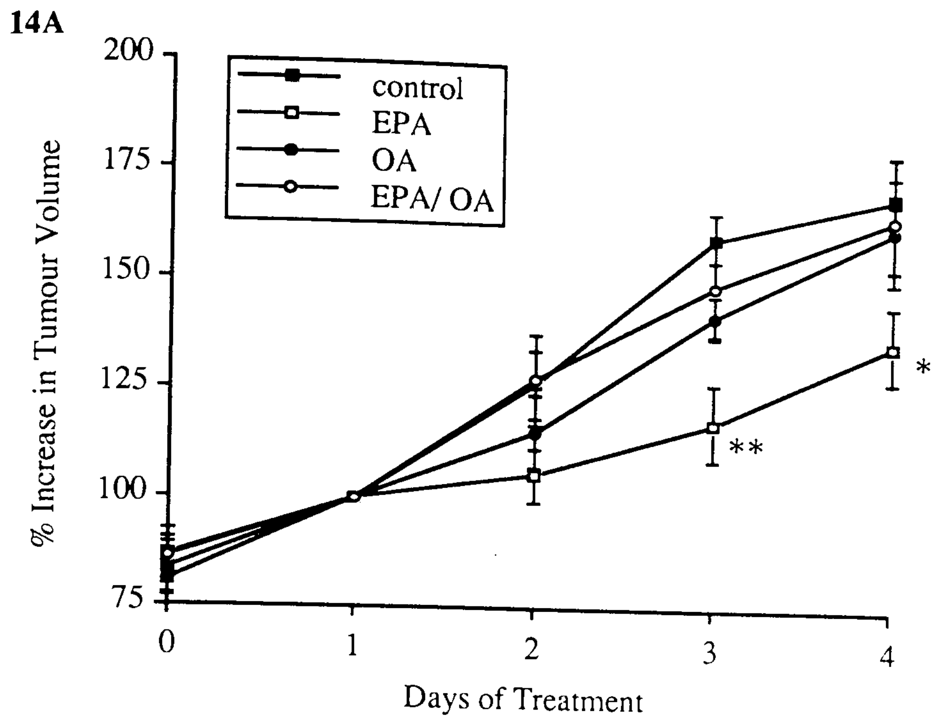


Figure 13. Effect of the n-3 PUFAs EPA (2.0g/kg) and DHA (2.25g/kg) on tumour growth (A) and weight loss (B) of MAC16 tumour-bearing mice.
Results are expressed as mean ± SEM.
** significantly different from control ($p<0.01$) by two-way ANOVA followed by Tuckey's Test.

14A



14B

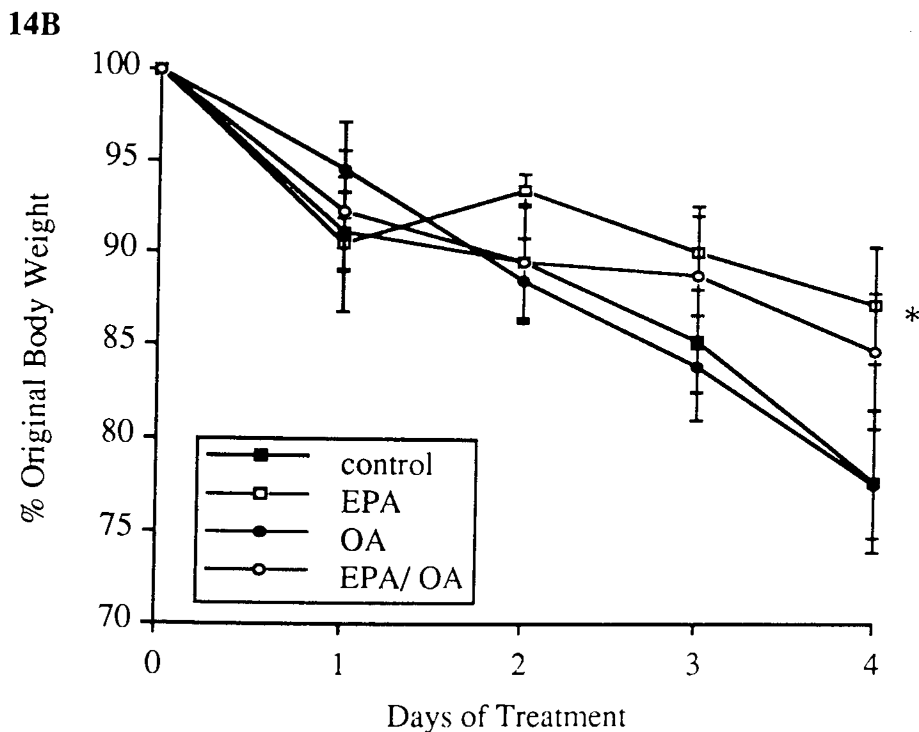
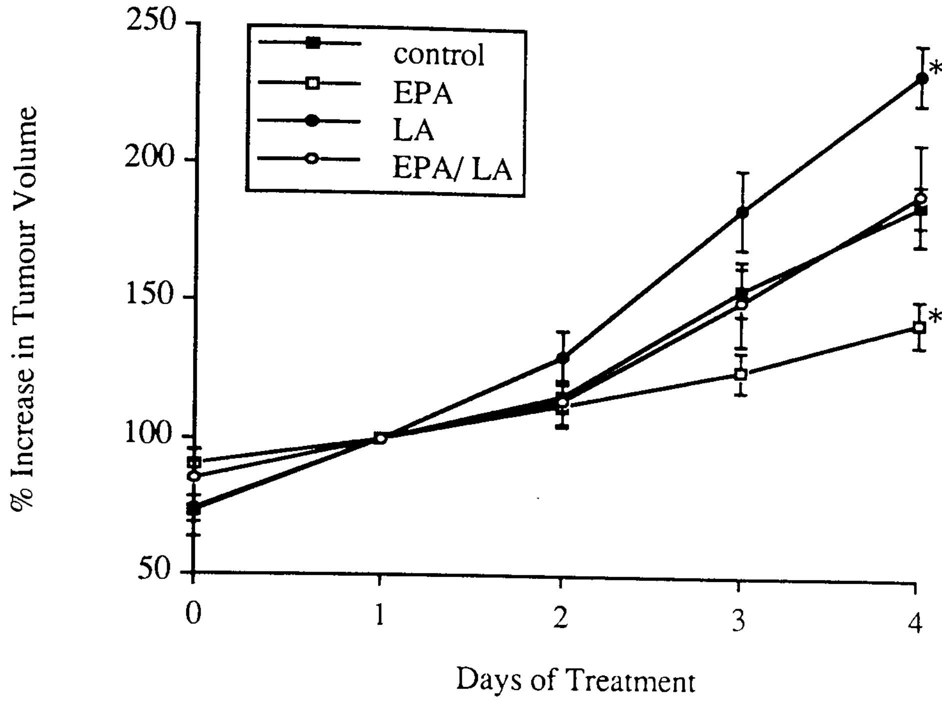


Figure 14. Effect of EPA (2.25g/kg), OA (2.0g/kg) and EPA + OA on tumour growth (A) and weight loss (B) of MAC16 tumour-bearing mice. Results are expressed as mean ± SEM for 6 animals per group. Significantly different from control * ($p<0.05$), ** ($p<0.01$) by two-way ANOVA followed by Tuckey's test.

15A



15B

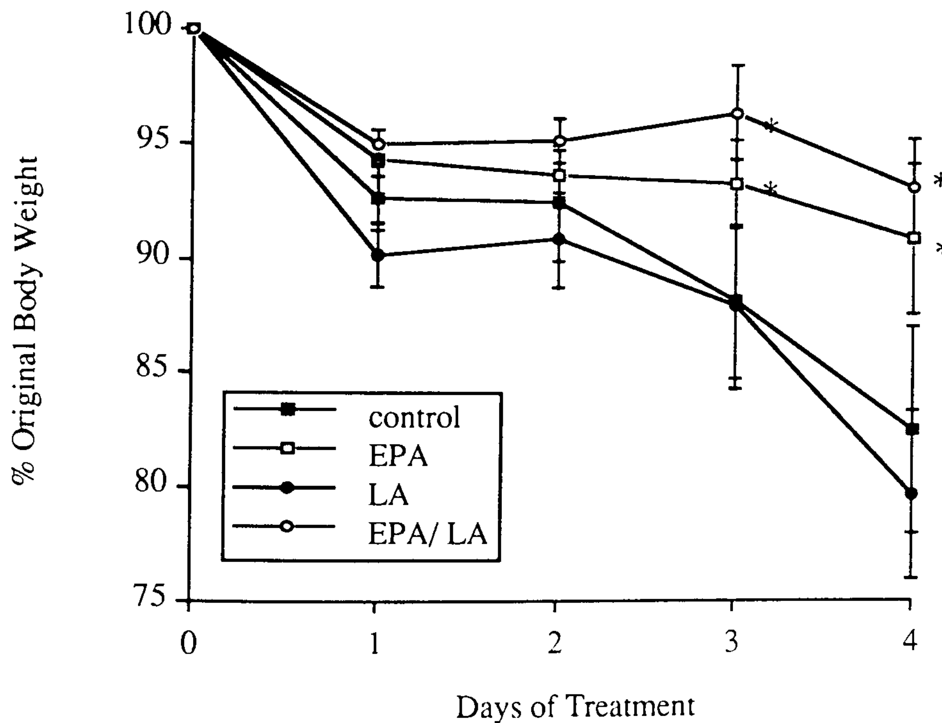
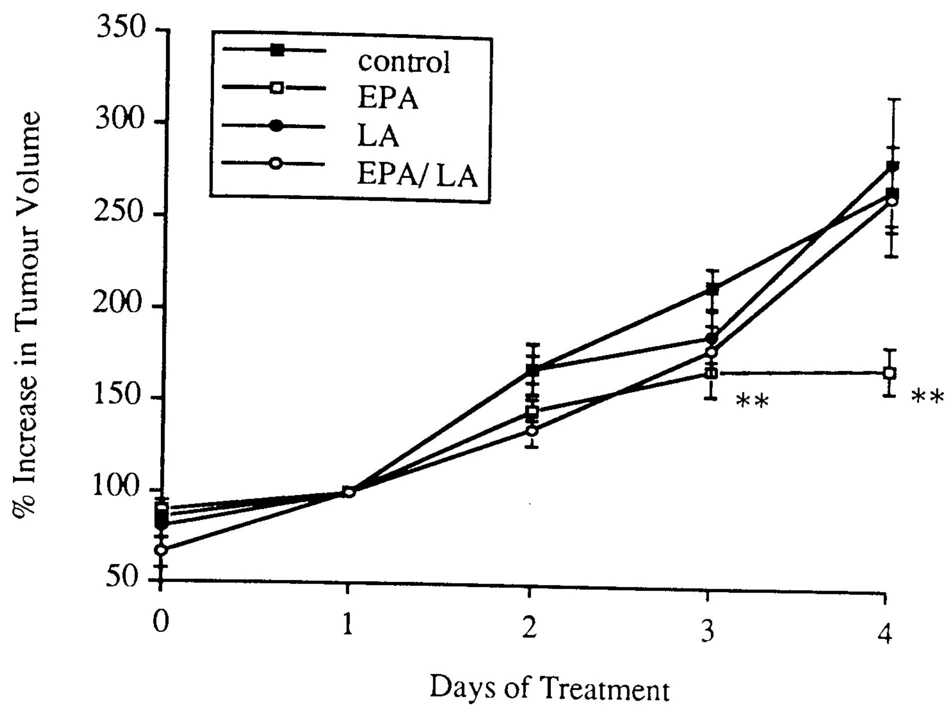
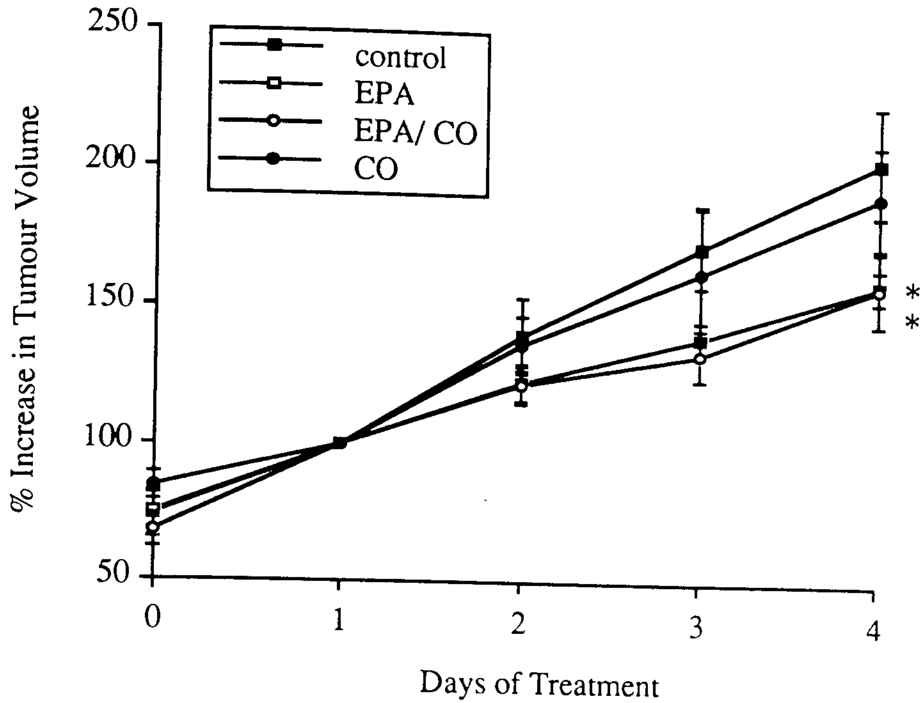


Figure 15. Effect of EPA (2.0g/kg), LA (1.9g/kg) and EPA + LA on tumour growth (A), (C), and weight loss (B) of MAC16 tumour-bearing mice. Results are expressed as mean ± SEM for 6 animals per group. Significantly different from control * ($p<0.05$), ** ($p<0.01$) by two-way ANOVA followed by Tuckey's test.

15C



16A



16B

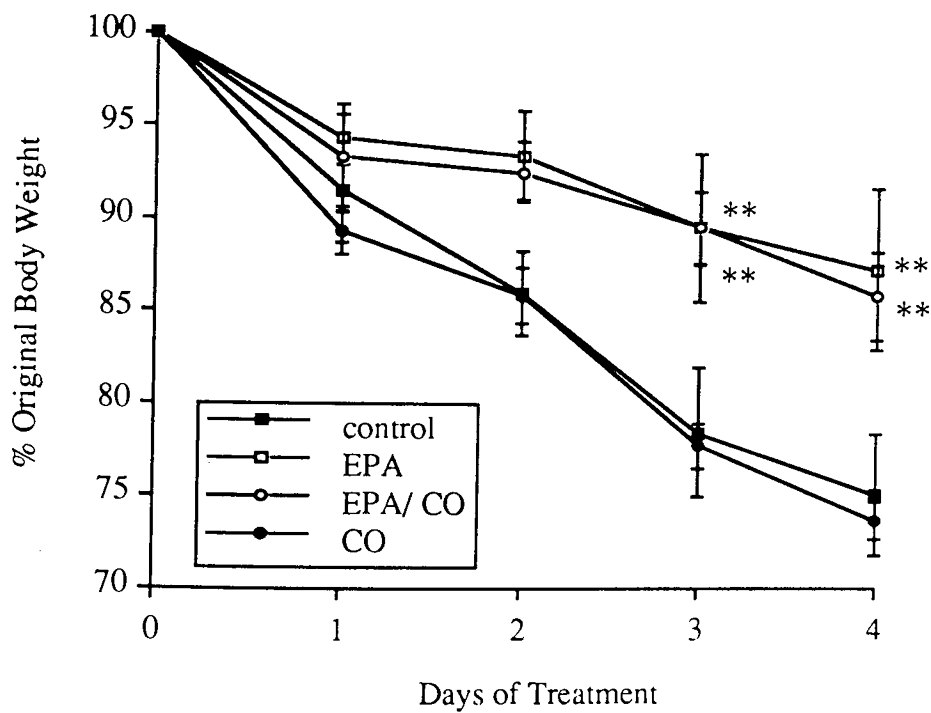


Figure 16. Effect of EPA (2.25g/kg), CO (5.0g/kg), and EPA + CO on tumour growth (A), and weight loss (B) of MAC16 tumour-bearing mice. Results are expressed as mean ± SEM for 6 animals per group. Significantly different from control * ($p<0.05$), ** ($p<0.01$) by two-way ANOVA followed by Tuckey's test.

The fatty acid composition of tissues from MAC16 tumour-bearing mice for each of these treatments are shown in Tables 3-5. Levels of EPA were found to be increased in both plasma, liver and tumour after oral administration of the PUFA. EPA and DHA were both found to cause a decrease in AA tumour concentration, and also in plasma AA concentration (Table 3A and C; 4A and B; 5B). Administration of LA resulted in elevation of plasma LA and tumour AA levels (Table 3A and C), while with the combination treatment of EPA and LA, plasma and tumour levels of both LA and AA were found not to be significantly different from those of control animals.

Administration of OA resulted in increased plasma concentration of OA which was found to be reversed by the concurrent administration of EPA (Table 4A), while animals dosed with CO did not show elevation of either of its two main constituents, LA and OA in plasma or tumour (Table 5A and B).

Food and water intake was not found to vary between control animals and those receiving LA, EPA or EPA/LA combination (Table 6) confirming that the anti-cachectic activity of EPA is not due to an increase in calorie consumption. Fatty acid composition of the rat and mouse breeding diet and corn oil is shown in Table 7.

Pooled results from the three sets of data for plasma of tumour-bearing and non tumour-bearing animals showed that the SA:OA ratio is increased in the tumour-bearing state (Fig. 17), supporting results presented in section 4.1, EPA treatment was found to have no effect on this ratio.

3A

Fatty Acid	non TB	TB	TB + EPA	TB + LA	TB + LA + EPA	TB + DHA
16:0	0.189 ± 0.014 *	0.135 ± 0.009	0.136 ± 0.014	0.136 ± 0.005	0.128 ± 0.014	0.133 ± 0.009
18:0	0.049 ± 0.010	0.061 ± 0.010	0.035 ± 0.014	0.047 ± 0.008	0.054 ± 0.008	0.063 ± 0.005
18:1 (n-9)	0.075 ± 0.013	0.046 ± 0.001	0.037 ± 0.013	0.036 ± 0.017	0.036 ± 0.005	0.040 ± 0.003
18:2 (n-6)	0.220 ± 0.014 *	0.149 ± 0.026	0.112 ± 0.027	0.365 ± 0.041 *	0.224 ± 0.031	0.147 ± 0.015
20:4 (n-6)	0.025 ± 0.004 *	0.044 ± 0.007	0.011 ± 0.003 *	0.034 ± 0.007	0.033 ± 0.005	0.016 ± 0.001 *
20:5 (n-3)	0.001 ± 0.001	0 ± 0	0.082 ± 0.017 *	0.005 ± 0.005	0.057 ± 0.015 *	0.004 ± 0.0005
22:6 (n-3)	0.008 ± 0.004	0.017 ± 0.002	0.004 ± 0.004	0.010 ± 0.006	0.006 ± 0.003	0.039 ± 0.003 *

Table 3A. Effect of the tumour-bearing state (TB) and treatment with EPA, LA, EPA/ LA and DHA on the fatty acid composition of total plasma lipids.
Results are expressed as mean ± SEM (mg fatty acid/ml plasma)
*significantly different from TB ($p<0.01$) by one-way ANOVA followed by Tuckey's test.

3B

Fatty Acid	non TB	TB	TB + EPA	TB + LA	TB + LA + EPA	TB + DHA
16:0	0.310 ± 0.030 *	0.540 ± 0.040	0.440 ± 0.009	0.280 ± 0.020	0.360 ± 0.030	0.580 ± 0.070
18:0	0.220 ± 0.030	0.380 ± 0.040	0.360 ± 0.070	0.210 ± 0.010	0.240 ± 0.020	0.470 ± 0.040
18:1 (n-9)	0.100 ± 0.020	0.180 ± 0.020	0.190 ± 0.040	0.110 ± 0.010	0.140 ± 0.010	0.230 ± 0.040
18:2 (n-6)	0.280 ± 0.020	0.330 ± 0.060	0.310 ± 0.080	0.210 ± 0.010	0.280 ± 0.020	0.500 ± 0.040
20:4 (n-6)	0.170 ± 0.010	0.190 ± 0.040	0.190 ± 0.040	0.140 ± 0.003	0.120 ± 0.004	0.150 ± 0.010
20:5 (n-3)	0.002 ± 0.002	0.019 ± 0.004	0.031 ± 0.008 *	0.004 ± 0.001	0.016 ± 0.002	0.019 ± 0.009
22:6 (n-3)	0.148 ± 0.019	0.301 ± 0.022	0.272 ± 0.062	0.157 ± 0.021	0.198 ± 0.012	0.504 ± 0.021 *

Table 3B. Effect of the tumour-bearing state (TB) and treatment with EPA, LA, EPA/ LA and DHA on the fatty acid composition of the major liver lipids.
Results are expressed as mean ± SEM (mg fatty acid/g liver)
*significantly different from TB ($p<0.01$) by one-way ANOVA followed by Tuckey's test.

3C

Fatty Acid	TB	TB + EPA	TB + LA	TB + LA + EPA	TB + DHA
16:0	0.892 ± 0.047	0.783 ± 0.039	0.770 ± 0.160	0.786 ± 0.051	0.971 ± 0.070
18:0	0.821 ± 0.061	0.826 ± 0.043	0.867 ± 0.087	0.758 ± 0.039	0.845 ± 0.067
18:1 (n-9)	0.920 ± 0.019	0.840 ± 0.087	0.945 ± 0.087	1.013 ± 0.010	1.515 ± 0.127*
18:2 (n-6)	0.630 ± 0.080	0.560 ± 0.040	0.630 ± 0.040	0.600 ± 0.010	0.750 ± 0.090
20:4 (n-6)	0.427 ± 0.005	0.310 ± 0.005*	0.051 ± 0.016*	0.340 ± 0.025	0.275 ± 0.029*
20:5 (n-3)	0.019 ± 0.001	0.049 ± 0.009*	0.019 ± 0.001	0.040 ± 0.003*	0.026 ± 0.005
22:6 (n-3)	0.263 ± 0.028	0.266 ± 0.010	0.242 ± 0.009	0.255 ± 0.017	0.530 ± 0.057*

Table 3C. Effect of treatment of MAC16 tumour-bearing mice (TB) with EPA, LA, EPA/ LA and DHA on the fatty acid composition of the major tumour lipids.
Results are expressed as mean ± SEM (mg fatty acid/g tumour)
*significantly different from TB ($p<0.01$) by one-way ANOVA followed by Tuckey's test..

4A

Fatty Acid	non TB	TB	EPA	OA	EPA + OA
16:0	0.380 ± 0.020	0.430 ± 0.040	0.290 ± 0.060	0.440 ± 0.040	0.320 ± 0.03
18:0	0.170 ± 0.008	0.160 ± 0.020	0.130 ± 0.040	0.170 ± 0.040	0.100 ± 0.010
18:1 (n-9)	0.290 ± 0.020	0.190 ± 0.040	0.140 ± 0.030	0.320 ± 0.070*	0.130 ± 0.010
18:2 (n-6)	0.520 ± 0.030	0.410 ± 0.100	0.330 ± 0.060	0.390 ± 0.080	0.210 ± 0.030
20:4 (n-6)	0.120 ± 0.020	0.100 ± 0.040	0.040 ± 0.008	0.150 ± 0.06	0.020 ± 0.009
20:5 (n-3)	0.010 ± 0.003	0.010 ± 0.010	0.090 ± 0.030*	0.020 ± 0.009	0.040 ± 0.010
22:6 (n-3)	0.080 ± 0.006	0.070 ± 0.008	0.080 ± 0.005	0.050 ± 0.009	0.060 ± 0.005

Table 4A. Effect of tumour-bearing state (TB) and treatment with EPA, OA and EPA + OA on the fatty acid composition of total plasma lipids.
Results are expressed as mean ± SEM (mg fatty acid/ml plasma).
*significantly different from TB ($p<0.01$) by one-way ANOVA followed by Tuckey's test.

4B

Fatty Acid	TB	EPA	OA	EPA + OA
16:0	1.640 ± 0.490	1.530 ± 0.180	1.780 ± 0.180	1.300 ± 0.120
18:0	1.850 ± 0.280	1.410 ± 0.040	1.770 ± 0.110	1.400 ± 0.060
18:1 (n-9)	3.640 ± 0.580	2.670 ± 0.250	3.740 ± 0.410	2.690 ± 0.190
18:2 (n-6)	1.850 ± 0.340	1.210 ± 0.110	1.980 ± 0.280	1.140 ± 0.140
20:4 (n-6)	0.880 ± 0.150	0.490 ± 0.030*	0.860 ± 0.130	0.460 ± 0.030*
20:5 (n-3)	0.053 ± 0.020	0.210 ± 0.020*	0.008 ± 0.008	0.230 ± 0.020*
22:6 (n-3)	0.580 ± 0.020	0.560 ± 0.050	0.680 ± 0.140	0.540 ± 0.040

Table 4B. Effect of treatment of MAC16 tumour-bearing mice (TB) with EPA, OA and EPA + OA on the fatty acid composition of the major tumour lipids.
Results are expressed as mean ± SEM (mg fatty acid/g tumour).
*significantly different from TB ($p<0.01$) by one-way ANOVA followed by Tuckey's test.

5A

Fatty Acid	non TB	TB	EPA	CO	EPA + CO
16:0	0.380 ± 0.020	0.400 ± 0.020	0.310 ± 0.050	0.370 ± 0.050	0.340 ± 0.030
18:0	0.170 ± 0.008	0.170 ± 0.020	0.170 ± 0.030	0.150 ± 0.020	0.130 ± 0.007
18:1 (n-9)	0.290 ± 0.020	0.240 ± 0.030	0.180 ± 0.030	0.210 ± 0.060	0.130 ± 0.009
18:2 (n-6)	0.520 ± 0.030	0.460 ± 0.020	0.410 ± 0.080	0.500 ± 0.110	0.280 ± 0.020
20:4 (n-6)	0.120 ± 0.020	0.110 ± 0.003	0.080 ± 0.007	0.110 ± 0.030	0.070 ± 0.007
20:5 (n-3)	0.010 ± 0.003	0.010 ± 0.0003	0.140 ± 0.003*	0.005 ± 0.003	0.050 ± 0.010* [a]
22:6 (n-3)	0.080 ± 0.006	0.060 ± 0.008	0.070 ± 0.020	0.030 ± 0.020	0.040 ± 0.005

Table 5A. Effect of tumour-bearing state (TB) and treatment with EPA, CO and EPA + CO on the fatty acid composition of total plasma lipids.
Results are expressed as mean ± SEM (mg fatty acid/ml plasma).
Significantly different from TB *($p<0.01$), and from TB+ EPA [a]($p<0.01$) by one-way ANOVA followed by Tuckey's test.

5B

Fatty Acid	TB	EPA	CO	EPA + CO
16:0	2.270 ± 0.190	1.820 ± 0.180	1.820 ± 0.140	2.080 ± 0.220
18:0	1.790 ± 0.070	1.740 ± 0.150	2.000 ± 0.340	1.910 ± 0.160
18:1 (n-9)	3.410 ± 0.400	2.940 ± 0.310	2.550 ± 0.320	3.310 ± 0.290
18:2 (n-6)	1.710 ± 0.140	1.480 ± 0.260	1.780 ± 0.240	2.090 ± 0.230
20:4 (n-6)	0.840 ± 0.040	0.580 ± 0.060*	0.890 ± 0.170	0.620 ± 0.040*
20:5 (n-3)	0.010 ± 0.010	0.280 ± 0.030*	0.060 ± 0.010	0.260 ± 0.030*
22:6 (n-3)	0.400 ± 0.140	0.540 ± 0.030	0.610 ± 0.080	0.610 ± 0.030

Table 5B. Effect of treatment of tumour-bearing mice (TB) with EPA, CO and EPA + CO on the fatty acid composition of the major tumour lipids.
Results are expressed as mean ± SEM (mg fatty acid/g tumour).
*significantly different from TB ($p<0.01$) by one-way ANOVA followed by Tuckey's test.

Treatment Group	Food Intake (cal/mouse/day)	Water Intake (ml/mouse/day)
control	9.6 ± 1.0	4.00 ± 0.18
EPA	9.6 ± 1.5	4.00 ± 0.28
LA	10.5 ± 1.9	4.17 ± 0.40
EPA/ LA	9.5 ± 1.5	3.75 ± 0.53

Table 6. Effect of EPA and LA on food and water intake of MAC16 tumour-bearing mice.
Results are expressed as mean ± SEM for 5 animals per group.

Fatty Acid	Rat and Mouse Breeding Diet (mg/g)	Corn Oil (%)
16:0	2.16	11.5
18:0	0.42	-
18:1 (n-9)	3.90	28.2
18:2 (n-6)	8.82	60.3
18:3 (n-6)	0.72	-
Total	16.02	100.0

Table 7. Long chain fatty acid composition of rat and mouse breeding diet and corn oil as determined by gas chromatography.

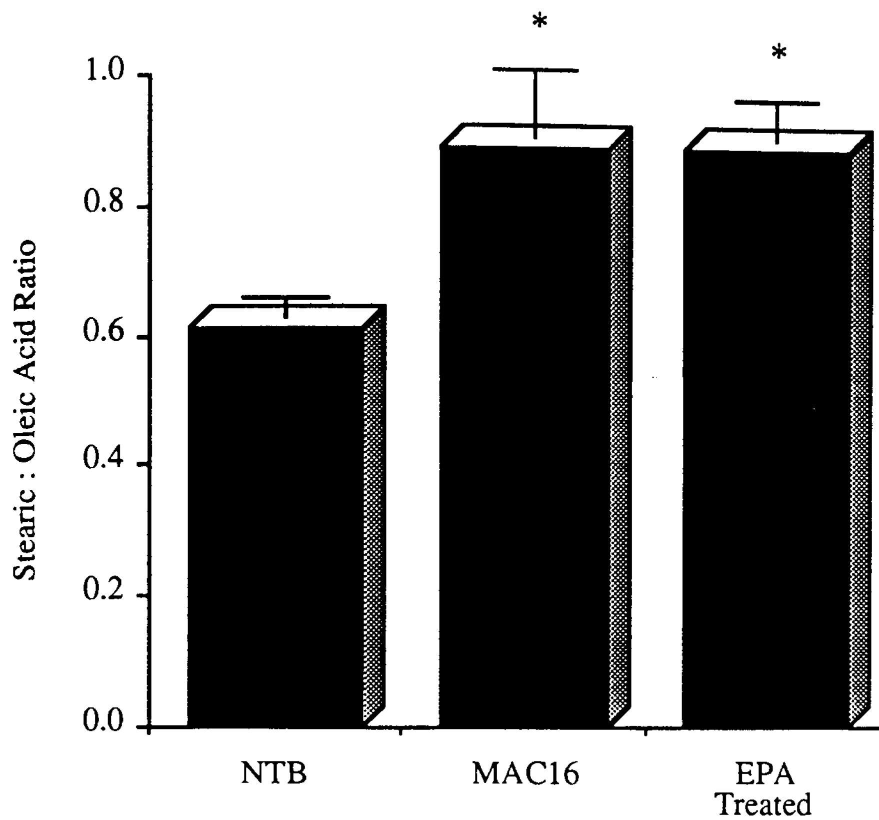


Figure 17. Effect of MAC16 tumour-bearing state, and EPA treatment on the plasma stearic : oleic acid ratio, compared with non tumour-bearing (NTB) controls.
Results are expresed as mean ± SEM (pooled from three sets of data).
* significantly different from control ($p<0.01$) by one-way ANOVA followed by Tuckey's test.

4.2.3 Discussion.

These data support the hypothesis that the anti-tumour and anti-cachectic activity of EPA are exerted via different mechanisms, as the tumour growth inhibition but not the reduction in weight loss caused by EPA was found to be reversed by the administration of either LA or OA.

The reversal by both LA and OA of the EPA induced tumour growth inhibition was not accompanied by decreased incorporation of EPA into the tumour, suggesting that the anti-tumour activity of EPA is indirect. Studies *in vitro* have shown inhibition by EPA of the activity of a MAC16 tumour-derived lipolytic factor at the site of the adipocyte. This effect was found to be due to inhibition of cAMP production by adenylate cyclase which may in part have been mediated by an inhibitory guanine nucleotide binding protein (Tisdale, 1993). This suggests that the indirect action of EPA on tumour growth inhibition may occur via the blockage of the lipolysis caused by the MAC16 tumour-derived factor, thereby decreasing the fatty acids available to the tumour from the circulation.

The stimulation of growth of the MAC16 by LA (Fig. 15) suggests that under normal dietary conditions the availability of this fatty acid may be a limiting factor. It would appear however, that at times the MAC16 tumour is able to bypass this limitation indicated by the failure of LA on occasion to further stimulate the growth of the MAC16 (Fig.15C).

The absence of any effect in response to the administration of CO (5.0g/kg) is reflected by the lack of an increase in either circulatory levels or tumour levels of either LA or OA, the two major components of CO. At the dose level of 5.0g/kg, it was calculated that mice dosed with the CO were receiving the equivalent of 3.01g/kg and 1.41g/kg of LA and OA respectively. It was therefore concluded that the lack of any increase in the fatty acid levels of tissues of mice dosed with the CO was due to differences between the pharmacokinetics of the uptake of triglycerides and free fatty acids.

Studies using the MAC26 adenocarcinoma (a tumour histologically related to the MAC16, but growing without the expression of cachexia) have shown that

maximal stimulation of tumour growth by LA occurs in mice receiving 4% of their total caloric intake (including 3% provided by the rat and mouse breeding diet) in the form of LA (Hussey, unpublished results, 1993). This agrees with the limit of dietary stimulation of carcinogenesis by essential fatty acids (EFA) reported by Roebuck *et al.* (1985) as 4-8% of the total energy intake of rats bearing azeserine induced pancreatic tumours. The average human population intake of LA as a percentage of total energy is reported by the British Nutrition Foundation task force (1993) as 6%, the recomended safe levels being in the range of 3-10%. Therefore it would appear that under normal dietary conditions human LA intake is above the threshold for maximal stimulation of carcinogenesis and tumour growth.

As both the n-3 fatty acids EPA and DHA caused a decrease in both plasma and tumour levels of AA, it can be concluded that EPA does not exert its anti-tumour or anti-cachectic effect directly via the inhibition of the desaturation of LA to AA, DHA having no activity against the MAC16 tumour.

There is known to be competition between the n-6 and n-3 series of fatty acids due to shared enzymes of desaturation, the n-3 series being generally thought to be favoured (Vance and Vance, 1991). Administration of fish oil has also been found to significantly inhibit activity of Δ9, Δ6 and Δ5 desaturases, resulting in a decrease in liver phospholipid content of AA (Christiansen *et al.*, 1991). In the same study, Δ6 desaturase activity was found to be stimulated by diets rich in LA. A similar decrease in AA content of brain and liver has also been reported in response to a fish oil diet (Bourre *et al.*, 1988). This is consistent with the decrease in AA found in the tumours of animals dosed with EPA in this study, although no change was observed in liver AA content.

It is not clear why the anti-cachectic/ anti-tumour effect of fish oil is specific to EPA while DHA is inactive, and one may speculate on several reasons for this difference. One possibility is that the added chain length of DHA results in a structural difference which precludes DHA from partaking in a cell surface receptor binding activity which is available to EPA. A second possibility is that the activity of EPA comes from a unique metabolite of the fatty acid; EPA biosynthesis via the

pathways of cyclooxygenase and lipoxygenase giving rise to a much greater variety of products than are formed from DHA (Willis, 1987). A third consideration in this context must be the varied effect of PUFAs on angiogenesis. Omental adipose tissue has been found to stimulate angiogenesis in the rabbit cornea (Silverman *et al.*, 1988; Goldsmith *et al.*, 1984). Recently the factor responsible for this has been identified as D[12(R)]-hydroxy-5,8,14-eicosatrienoic acid, a cytochrome P450-dependent arachidonate metabolite (Masferrer *et al.*, 1989; 1991). In another study, Murota *et al.* (1992) showed angiogenesis *in vitro* in response to AA, but also found that EPA possessed anti-angiogenic properties, while DHA was found to be inactive. This observation may have relevance to the results described in this study, as changes in angiogenic processes in response to fatty acids may affect tumour growth, or the release of tumour-produced factors.

4.3 Cell Kinetics of MAC16 and MAC26 Tumour Growth and Modulation by PUFAs.

4.3.1 Introduction.

In contrast to the exclusivity of the anti-tumour and anti-cachectic activity of EPA *in vivo*, *in vitro* both EPA and DHA have cytotoxic activity against the MAC16 cell line (Tisdale and Beck, 1991). These results lead to the suggestion that *in vivo* EPA may not act as an anti-proliferative agent. This hypothesis is investigated by examining the cell kinetics of tumour growth inhibition by EPA. The mode of action of LA stimulation of tumour growth and its reversal of EPA induced tumour growth inhibition is also investigated.

Tumour cell loss (ϕ) and potential doubling time (T_p) were calculated using the direct method of measuring the decrease in the specific activity of radioactivity in tumours labelled with [^{125}I]iododeoxyuridine (^{125}I-Udr) (Gabor *et al.*, 1985; Gabor and Abraham, 1986). The ^{125}I-Udr method is based on the similarity in size between an iodine and methyl group, the ^{125}I-Udr therefore being incorporated into DNA during replication (Steel, 1977).

An alternative procedure for the calculation of cell kinetics consists of an indirect method utilising [^{3}H]thymidine (^{3}H-Tdr) incorporation into tumours, and computer aided analysis of fraction of labelled mitoses (FLM) curves (Gabor *et al.*, 1985). These two techniques have been compared by several workers who have reported a good general agreement between ^{125}I-Udr and ^{3}H-Tdr values for cell loss (Begg, 1977; Franko and Kallman, 1980; Gabor *et al.*, 1985). Begg (1977) however found that with high cell loss tumours, the ^{125}I-Udr technique gave lower values, implying reutilisation of the label by the tumours. Reutilisation can take two forms, either from the breakdown of dead tumour cells which then release the labelled base, or from the release of the label from other organs of the body and its subsequent uptake by the tumour from the blood stream. A third mechanism by

which a false reading may occur is via the influx of other labelled host cells, for example in response to inflamation (Franko and Kallman, 1980).

Despite this possible drawback of the ^{125}I-Udr method, it has been widely used and provides a more rapid and simpler method of cell loss determination than the ^{3}H-Tdr method, which itself is not devoid of possible sources of error (Steel, 1977). The ^{125}I-Udr labelling method was therefore judged to be suitable for determination of changes in ϕ of the MAC16 and 26 tumours after administration of fatty acids in these studies.

4.3.2 Results.

Data presented in Fig. 18 show the biphasic response of MAC16 tumour growth to the administration of EPA. Initially, EPA (2.0g/kg) results in a stationary phase of tumour growth, after which a phase of tumour regrowth occurs, at a rate similar to that seen in control tumour-bearing mice. Data obtained in cell kinetic studies for tumour doubling time (T_D), potential doubling time (T_P) and cell loss factor (ϕ) are presented in Table 8. The inhibitory effect of EPA appears to be due to an increase in cell loss from 38 to 71%, which is then decreased to 52% during the period of exponential growth.

The reversal of EPA induced tumour growth inhibition by LA (Fig. 19) was found to be associated with a decrease in cell loss from 71% to 45% (Table 8). LA itself was found not to affect either cell loss or potential doubling time - a measure of rate of cell proliferation.

An assessment of extent of necrosis in the MAC16 tumour during treatment with EPA found areas of necrotic tissue to be greater in tumours treated with EPA than in control tumours, and also appeared to increase over time with EPA treatment (Table 9) (studies carried out by Dr J. Double, Bradford University).

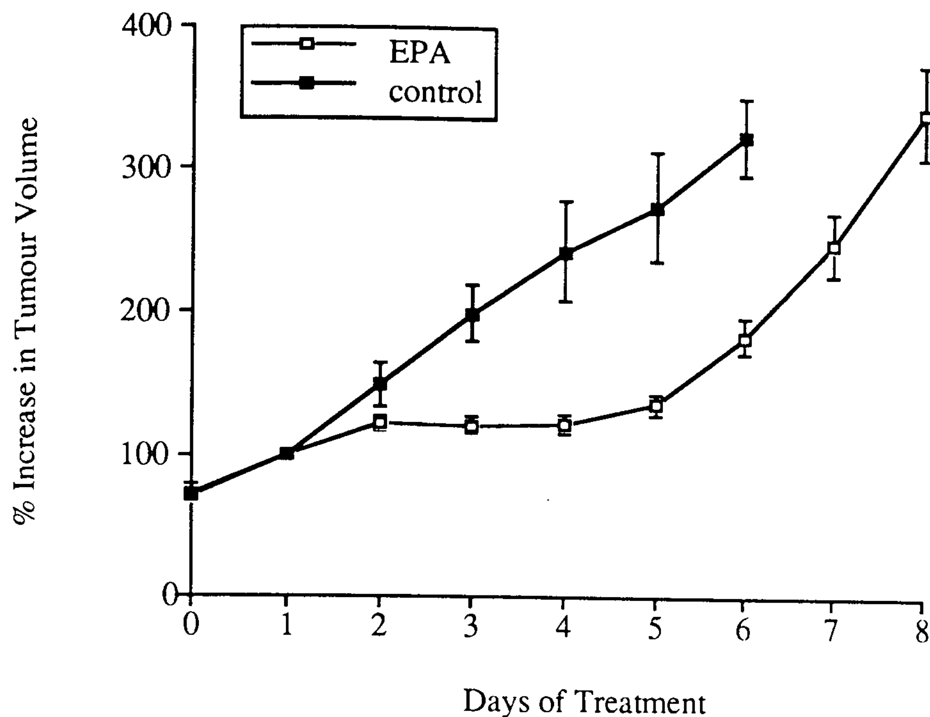


Figure 18. Biphasic response of MAC16 tumour growth to daily administration of EPA (2.0g/kg).
Results are expressed as mean ± SEM. n=24 for both groups on day 1, after which 4 mice were sacrificed daily from the control group. To determine the growth kinetics of both phase 1 and 2 of EPA treated tumour growth, 4 mice were sacrificed daily from day 1 or day 4 respectively in two separate experiments.

	T_D (hr)	T_P (hr)	ϕ (%)
MAC16			
control	50.0	30.0	38.0
EPA phase I	110.0	32.0	71.0
EPA phase II	55.0	26.0	52.0
LA	53.0	31.0	40.0
EPA/ LA	48.0	26.0	45.0
MAC26			
control	130.0	41.0	68.0
LA	84.0	28.0	67.0

Table 8. Cell kinetics of MAC16 and MAC26 tumours.

	Mean Area of Tumour (%) ± S.E.M.		
	Viable Tumour	Necrosis	Space
Control (1)	89.0 ± 7.0	10.0 ± 6.0	1.0 ± 1.0
EPA (50mg)			
Day 1	78.0 ± 3.0	20.0 ± 3.0	3.0 ± 2.0
Day 2	75.0 ± 2.0	23.0 ± 2.0 *	2.0 ± 0.5
Day 3	79.0 ± 3.0	20.0 ± 3.0	1.0 ± 0.2
Day 4	62.0 ± 8.0	36.0 ± 8.0	2.0 ± 1.0
Control (2)	69.2 ± 6.0	26.0 ± 6.0	5.0 ± 1.0
EPA (75mg) Day 10	39.0 ± 13.0 *	55.0 ± 11.0 *	5.0 ± 3.0

Table 9. Histological data pertaining to the MAC16 tumour; in experiment (1), before and during treatment with EPA (50mg), and in experiment (2), after 10 days of treatment with EPA (75mg) compared with control. * significantly different from respective control ($p<0.01$) by t-test.
(Data collected by Dr. J. Double, Bradford University).

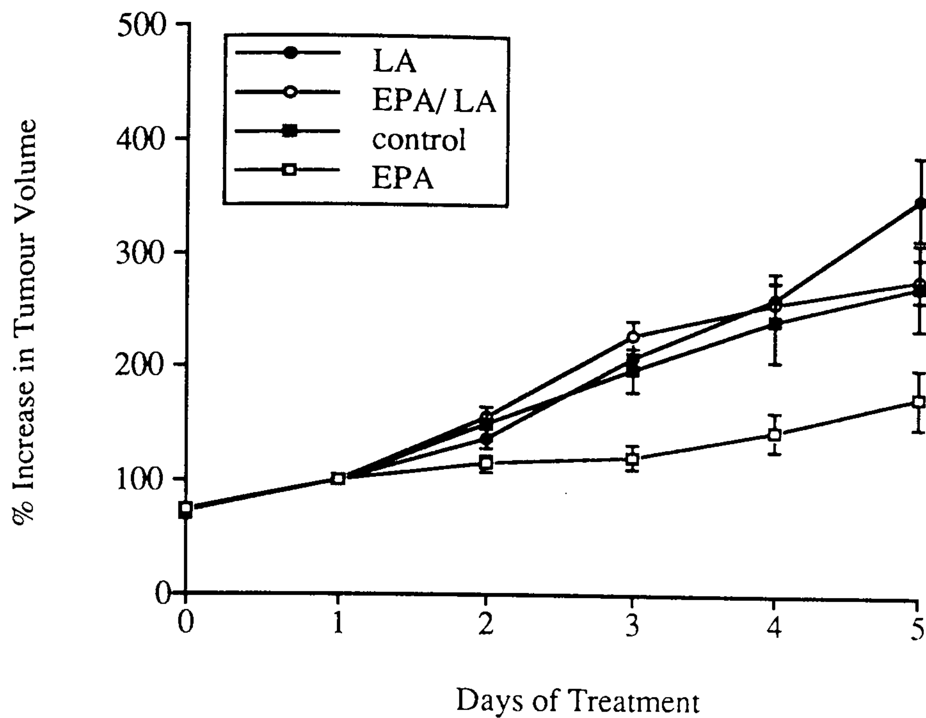


Figure 19. Effect of EPA (2.0g/kg), LA (1.9g/kg) and EPA + LA on growth of the MAC16 tumour.
Results are expressed as mean ± SEM. n=24 for each group on day 1, after which 4 mice were sacrificed from each group daily.

LA has also been found to stimulate growth of the MAC26 adenocarcinoma *in vivo* (Hussey, 1993 unpublished results), the MAC26 is histologically related to the MAC16 tumour, but grows without the expression of cachexia. The kinetic studies on the LA stimulation of MAC26 tumour growth indicated no effect of LA on cell loss in this model, while a reduction in potential doubling time of 33% occurred (from 41 to 28 hours) (Fig. 20, Table 8) (experiment carried out in collaboration with Ms H. Hussey, Aston University).

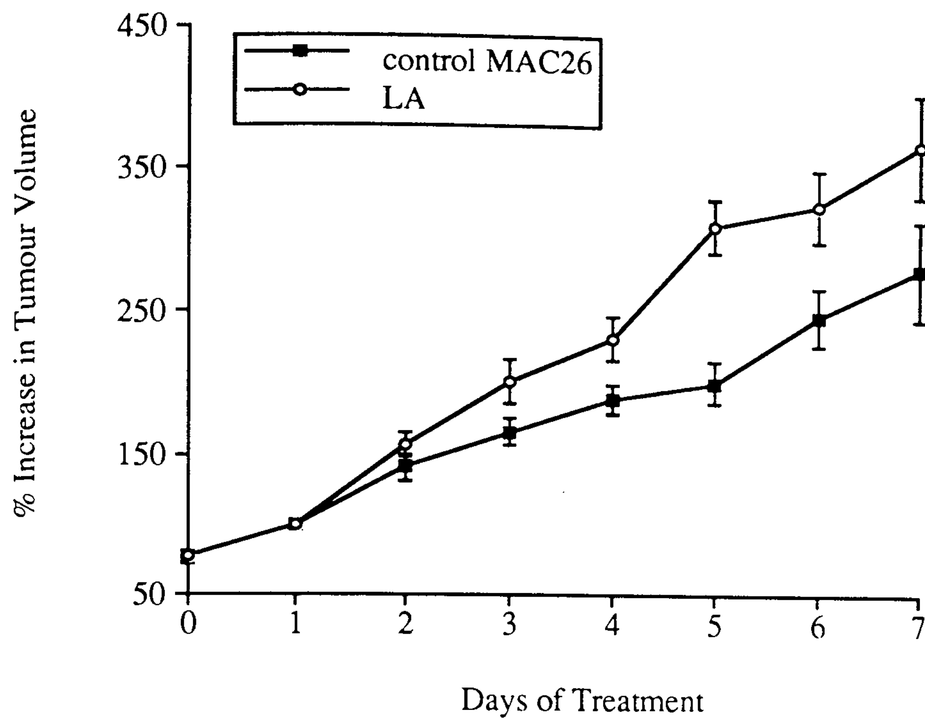


Figure 20. Effect of LA (2.37g/kg) on growth of the MAC26 tumour. Results are expresed as mean ± SEM. n=24 on day 1, 4 mice were sacrificed from each group from day 3.

4.3.3 Discussion.

The growth of tumours depends on a delicate balance between the rates of cell production and cell loss (Steel, 1977); for tumour growth to occur, cell production must exceed cell loss. As the cell loss factor approaches 100%, the two components are in equilibrium and neither growth nor regression of the tumour occurs.

The factor of cell loss obtained in this study for the MAC16 tumour (38%) was found to be similar to that of the CBA sarcoma (36%), and close to the range reported for the C3H mammary carcinoma (41-55%) by Begg (1977).

These data confirm the hypothesis that EPA does not exert its anti-tumour activity via inhibition of cell proliferation. The anti-tumour activity of EPA was shown to be biphasic, characterised by an initial stationary phase of tumour growth during which cell loss is increased to 71%, followed by a period of exponential growth similar to that of control growth during which cell loss is reduced to 52%. As we have previously shown (section 4.2) that the anti-tumour effect of EPA is indirect, these results suggest that EPA may act via inhibition of some process essential for the maintenance of tumour cell viability rather than by directly causing a cytotoxic event in tumour cells. Studies performed by Dr J. Double (Bradford University) (Table 9) have found increased areas of necrosis in tumours treated with EPA compared with control tumours, which suggests that EPA increases cell loss via necrosis rather than by induction of the mechanism of programmed cell death, apoptosis.

Reversal of EPA-induced tumour growth inhibition by LA was found to be associated with a decrease in cell loss from 71 to 45%, suggesting that LA may be involved in prolonging the life span of tumour cells, although administration of LA itself had no effect on either cell loss or proliferation in the MAC16 tumour. As previously indicated (section 4.2) the stimulatory effect of LA is dependent on the growth rate of the tumours; in this study growth stimulation by LA can be seen to have been minimal (Fig.19), this may explain why no effect on cell loss or potential doubling time was observed.

Results obtained with the slower growing MAC26 tumour show that LA is capable of reducing the potential doubling time of tumours, while having no effect on cell loss. Cell loss in the MAC26 tumour was found to be higher than for the MAC16 tumour (68% compared with 38%), which when combined with the slower rate of proliferation (T_P 41.3 hours, compared with 30 hours) explains the longer doubling time observed in this model. Interestingly, the potential doubling time of LA stimulated MAC26 tumour growth, 27.6 hours is very similar to the rates found for both control and LA treated MAC16 tumour growth (30 and 31 hours). This may indicate a maximal rate of tumour cell production for the MAC tumours under physiological conditions, and may explain why LA stimulation of tumour growth is not always seen. Under certain circumstances the MAC16 tumour may be capable of proliferating at a rate close to its maximum capacity, and therefore under these conditions LA is not limiting, supplementation with the fatty acid resulting in no further stimulation of tumour growth. Therefore a role is proposed for LA or one of its metabolites in both preservation of cell viability, and as a potential limiting factor for cell proliferation.

4.4 Effect of the Ethyl Esters of EPA and DHA on Tumour Growth and Weight Loss of MAC16 Tumour-bearing Mice.

4.4.1 Introduction.

The free fatty acid of EPA in the form of an 80%+ preparation has until recently been of limited availability due to prohibitive costs and problems of stability during the purification process. The ethyl ester of EPA (EEE) is however, comparatively widely available at a high purity being more stable than the free acid. EEE has also been found to compare favourably with a concentrated extract of fish oil as an anti-thrombotic agent (Wojenski *et al.*, 1991).

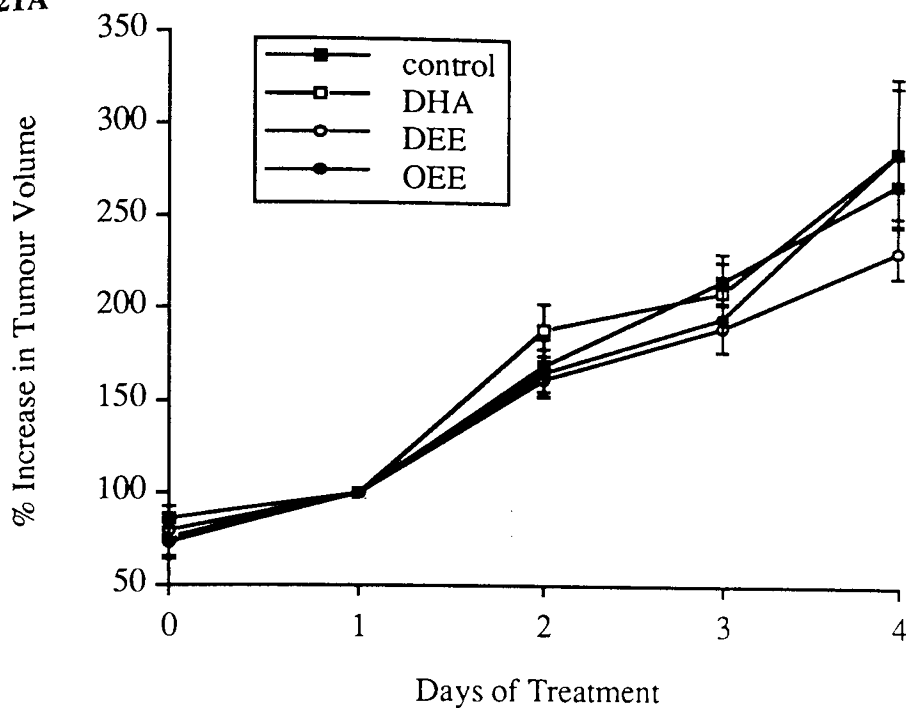
In this study, the effect of the ethyl esters of EPA and DHA on the tumour growth and cachexia of the MAC16 model was investigated.

4.4.2 Results.

Data presented in Fig.21 confirm previous findings that DHA is inactive against the MAC16 adenocarcinoma. Both the ethyl ester of DHA and olive oil were found to have no effect on tumor growth or weight loss of MAC16 tumour-bearing animals. The ethyl ester of EPA (2.5g/kg), unlike the free acid was also found to be inactive against this model (Fig.22).

Stimulation of esterase activity *in vivo* by the administration of a high fat diet has previously been found to enhance plasma levels of EPA after oral delivery of EEE (Lawson and Hughes, 1988a). In the study presented here, when given in combination with a high fat diet deriving 80% of the calories from medium chain triglycerides (MCT diet), weight loss of animals receiving EEE (2.5g/kg) was not found to differ from control animals ingesting either the MCT diet, or the rat and mouse breeding diet (RMB diet) (Fig.23B). At a higher dose (5.0g/kg), EEE treatment resulted in an increase in weight loss above the level of control mice.

21A



21B

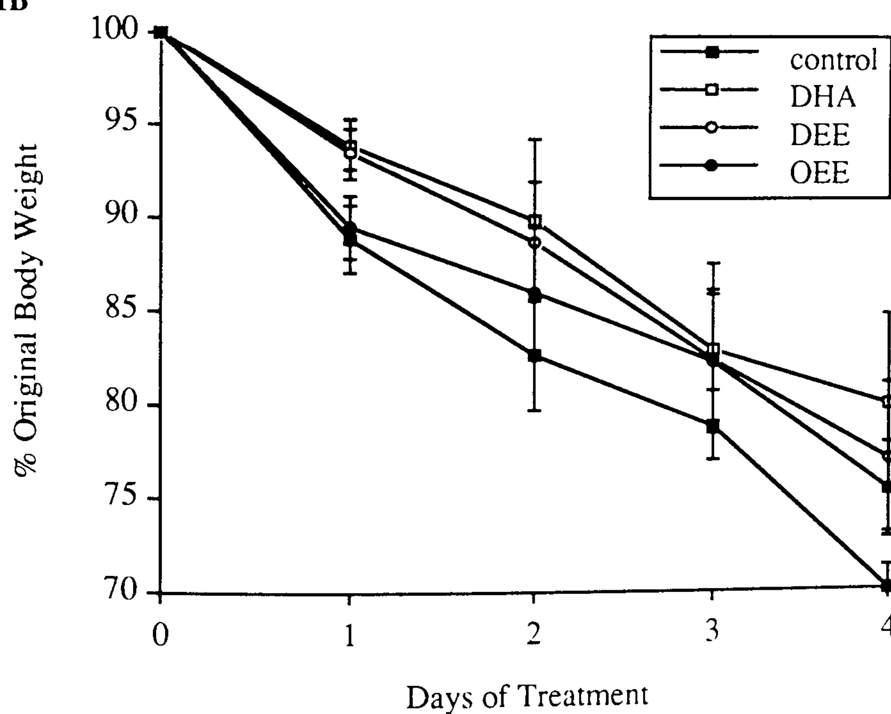
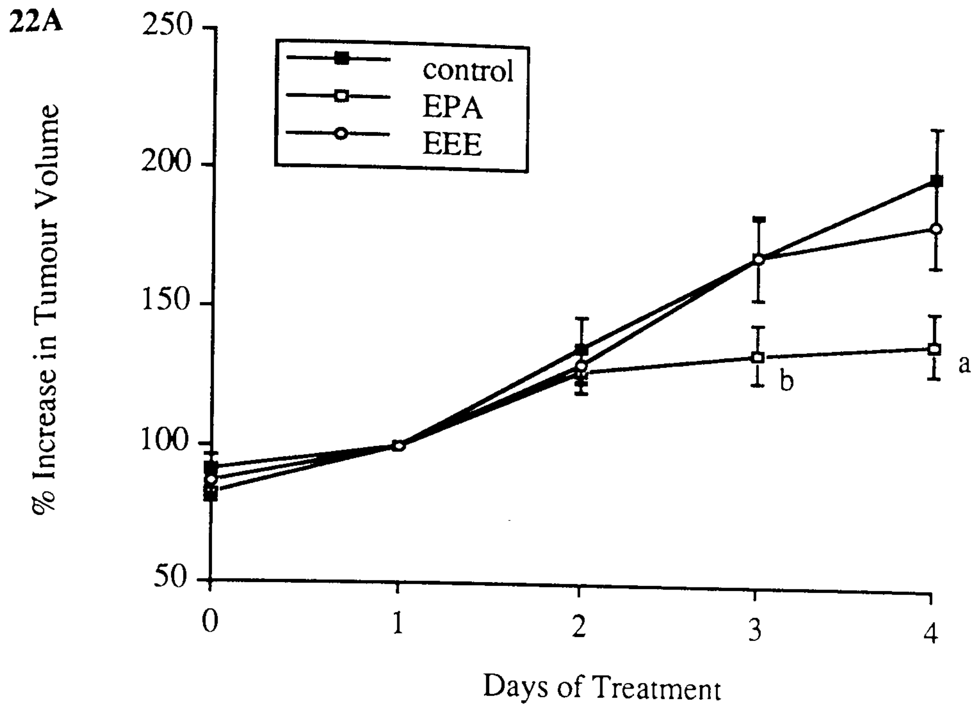


Figure 21. Effect of DHA (2.25g/kg) DEE (2.5g/kg) and OEE (2.5g/kg) on tumour growth (A) and weight loss (B) of MAC16 tumour-bearing mice. Results are expressed as mean ± SEM for 6 animals per group.

22A



22B

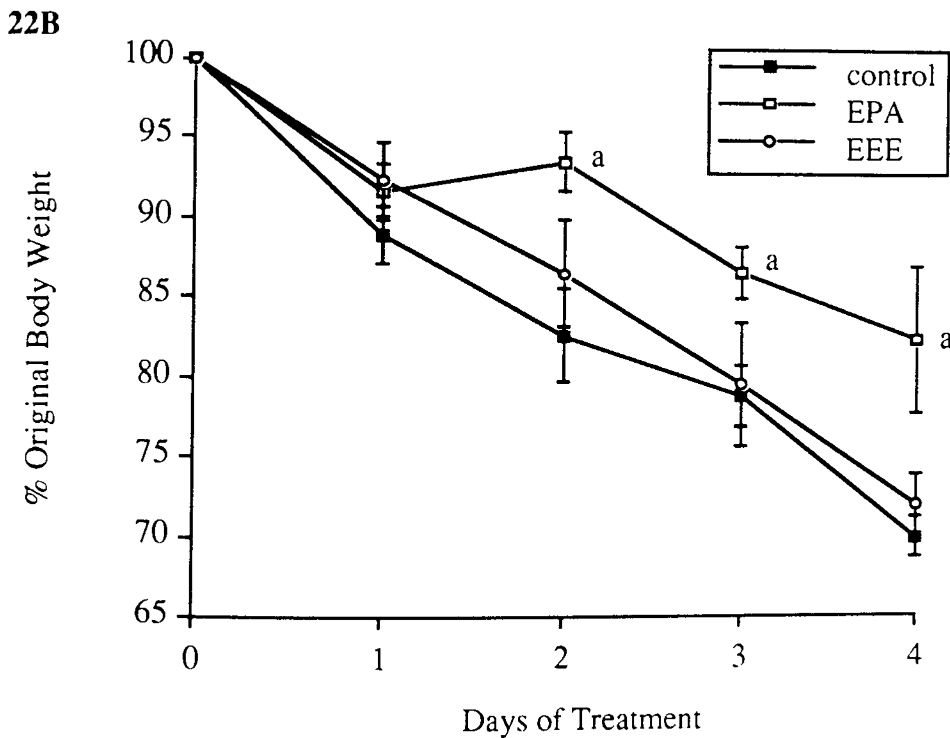
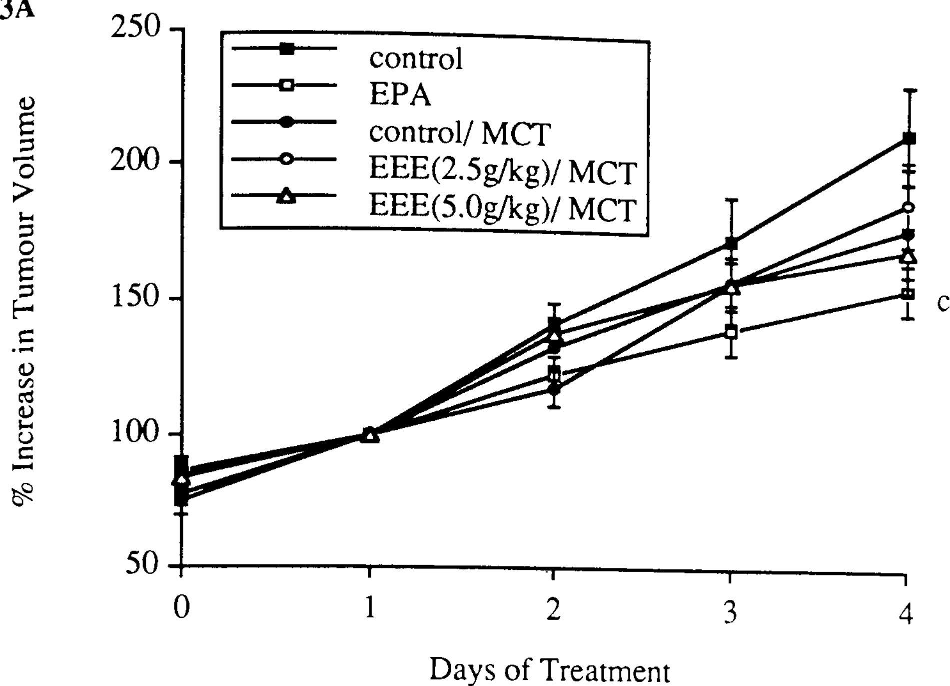


Figure 22. Effect of EPA (2.0g/kg) and EEE (2.5g/kg) on tumour growth (A) and weight loss (B) of MAC16 tumour-bearing mice.
Results are expressed as mean ± SEM for 6 animals per group.
Significantly different from control $^{a}(p<0.01)$ $^{b}(p<0.05)$ by two-way ANOVA followed by Tuckey's test.

23A



23B

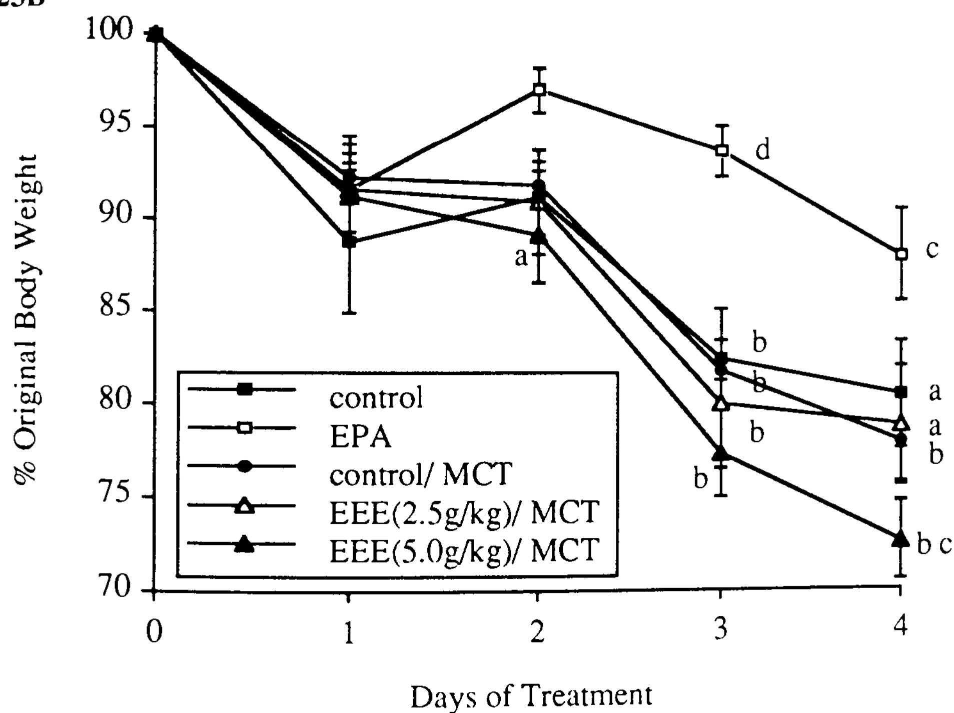


Figure 23. Effect of EPA (2.0g/kg), and EEE (2.5g/kg; 5.0g/kg) in combination with an MCT diet on tumour growth (A) and weight loss (B) of MAC16 tumour-bearing mice.

Results are expressed as mean ± SEM for 6 mice per group.

Significanly different from EPA [a]($p<0.05$) [b]($p<0.01$) and from control [c]($p<0.05$) [d]($p<0.01$) by two-way ANOVA followed by Tuckey's test.

The effect of EEE/ MCT diet treatment on tumour growth is less clear, both control animals receiving the MCT diet, and those receiving MCT plus EEE (2.5 or 5.0g/kg) were found to show reduced tumour growth when compared to control MAC16 tumour-bearing animals fed the RMB diet, although the inhibition was not found to be significant and did not occur to the same extent observed in response to EPA free acid treatment (Fig.23A). Food and water intake were found to be slightly depressed in animals fed the MCT diet (Table 10) although the differences were not significant, with the exception of the water intake of the MCT/ EEE (5.0g/kg) group. Long chain fatty acids were found to appear in the two diets in similar percentages, although the actual PUFA content of the MCT diet was found to be 44% of that of RMB diet (Table 11). These results indicate that the feeding of a high fat diet does not induce anti-tumour or anti-cachectic activity in response to EEE treatment.

Incubation of EEE with pig liver esterase for 24 hours was found to result in approximately 80% conversion to EPA free acid (by gas chromatography). The treatment also resulted however in the coagulation of the fatty acids into polymers which were not easily disrupted. It was therefore found to be impossible to form a homogeneous emulsion of the fatty acid in the incubation buffer. This caused severe problems in the oral administration of the mixture. Animals were dosed with 100μl of the mixture, equivalent to 2.5g/kg or 50mg fatty acid (20% EEE, 80% EPA by gas chromatography). The effects of the hydrolysed EEE mixture on tumour growth and weight loss of MAC16 tumour-bearing mice were found not to differ significantly from those obtained with either the untreated EEE or control tumour-bearing animals (Fig.24).

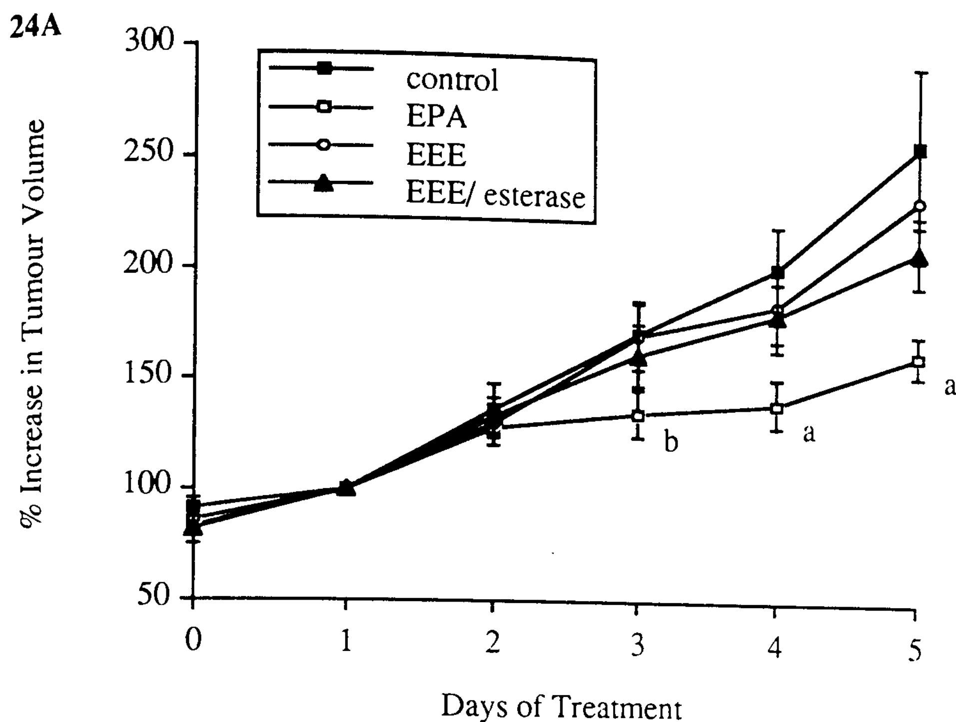
Treatment	Food Intake g/ mouse/ day	Water Intake ml/ mouse/ day
control MAC16	2.5 ± 0.4	3.0 ± 0.5
EPA(2.0g/kg)	2.7 ± 0.3	2.1 ± 0.1
control/ MCT	2.1 ± 0.6	2.2 ± 0.2
EEE(2.5g/kg)/ MCT	2.0 ± 0.5	2.2 ± 0.06
EEE(5.0g/kg)/ MCT	1.5 ± 0.3	1.3 ± 0.3 *

Table 10. Daily food and water intake of MAC16 tumour-bearing mice treated with EPA (2.0g/kg) and EEE (2.5g/kg; 5.0g/kg). *significantly different from control ($p<0.01$) by one-way ANOVA followed by Tuckey's test.

Fatty Acid	% of Total PUFAs		Concentration (mg/g)	
	RMB	MCT	RMB	MCT
16:0	13.5	19.7	2.16	1.40
18:0	2.6	10.6	0.42	0.75
18:1 (n-9)	24.3	14.8	3.90	1.05
18:2 (n-6)	55.1	50.7	8.82	3.60
18:3 (n-6)	4.5	4.2	0.72	0.30
Total	100.0	100.0	16.02	7.10

Table 11. PUFA composition of rat and mouse breeding diet (RMB) and medium chain triglyceride diet (MCT).

24A



24B

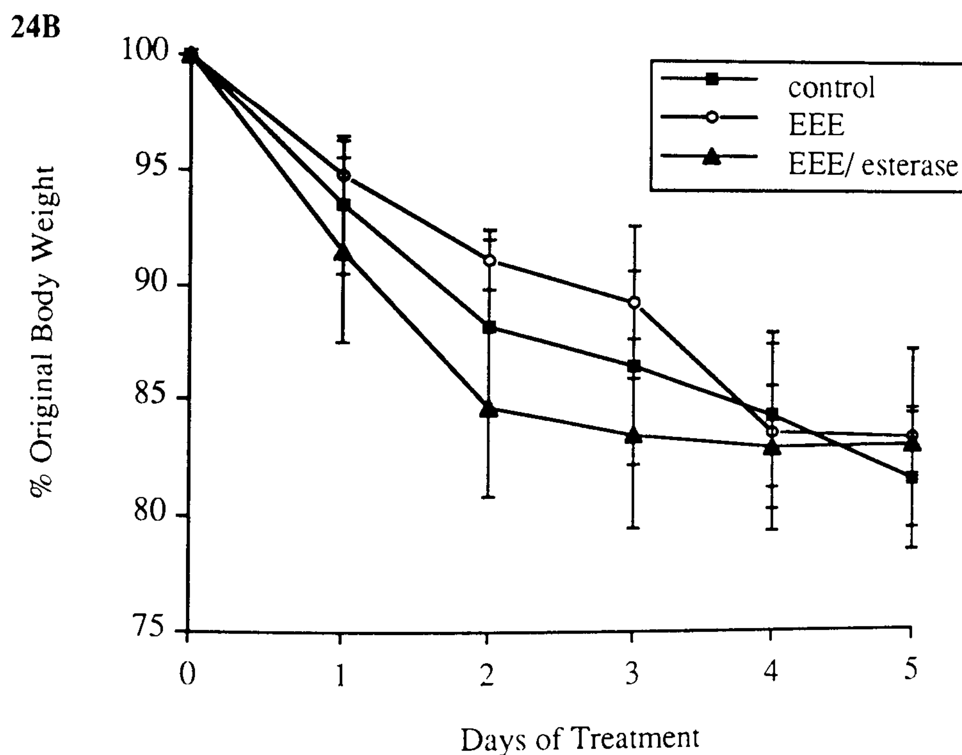


Figure 24. Effect of EEE pretreated with pig liver esterase on the tumour growth (A) and weight loss (B) of MAC16 tumour-bearing mice. Results are expressed as mean ± SEM for 6 animals per group. Significantly different from control [a]($p<0.01$) [b]($p<0.05$) by two-way ANOVA followed by Tuckey's test.

The pharmacokinetics of the uptake of these various PUFA treatments show a rise in plasma [EPA] by 6 hours after the initial dose (Fig.25). After 24 hours the level was found to have dropped although it still remained above the concentration of untreated mouse plasma. Subsequent treatment with EPA resulted in a more sustained increase in plasma [EPA], which at 48 hours was found to be significantly higher than plasma [EPA] in any other treatment group. After this point, plasma [EPA] gradually decreased despite further treatment with EPA. EEE (2.5g/kg) treatment resulted in a similar plasma [EPA] profile, the second peak however being of lower concentration, and occurring approximately 24 hours after that of the free acid treatment. The profile obtained for the esterase treated EEE was found to be almost identical to that of untreated EEE. Animals receiving EEE in conjunction with the MCT diet showed lower values of plasma [EPA] than EPA and EEE treatments for 72 hours after the initial treatment. The increase in plasma [EPA] over time however was found to be more sustained, and to occur continuously during treatment. After 96 hours, the EEE plus MCT group had significantly higher plasma [EPA] than the group receiving EEE alone.

Tumour EPA concentration 96 hours after initial treatment, was found to be increased above control, and control/ MCT group levels in all treatment groups. The EPA group showed significantly higher levels of EPA than either EEE or esterase treated EEE groups. Feeding of the MCT diet was also found to increase the level of EPA present in the tumour after treatment with EEE (Fig.26).

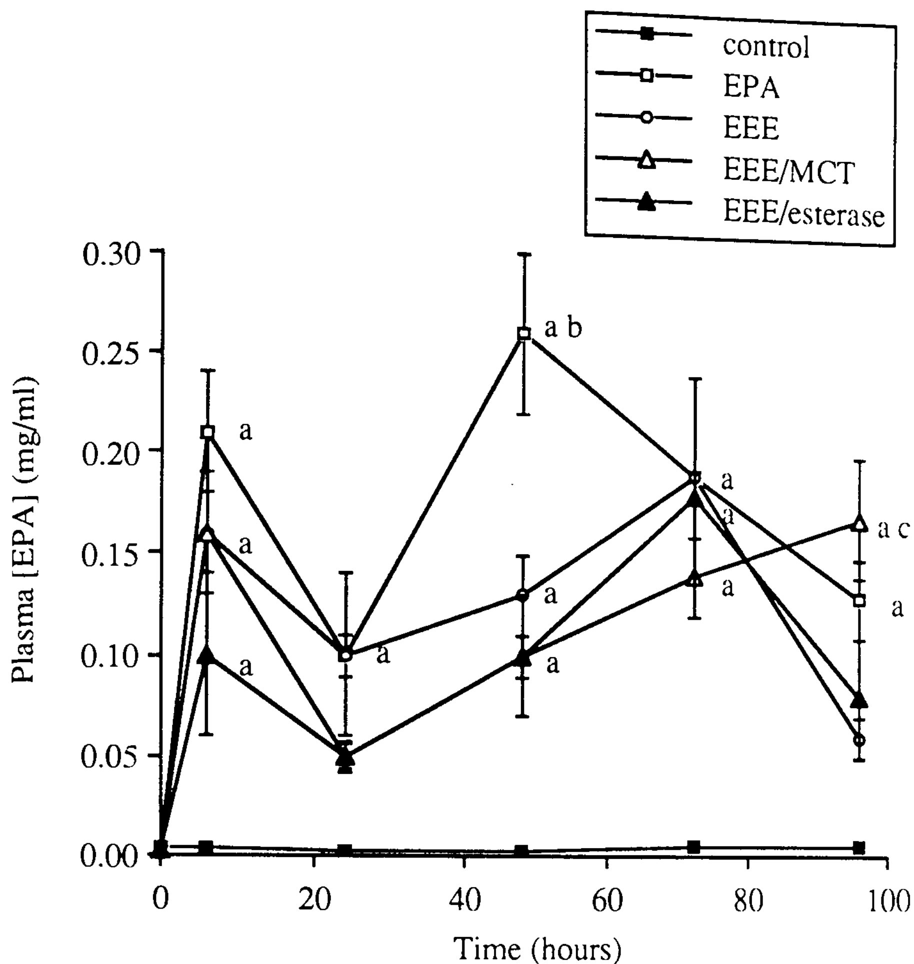


Figure 25. Plasma [EPA] after treatment with EPA (2.0g/kg), EEE (2.5g/kg), EEE (2.5g/kg) plus MCT diet and EEE pretreated with esterase.
Results are expresed as mean ± SEM for 4 animals per group.
Significantly different from control ($p<0.01$), from all other groups ($p<0.01$) and from EEE ($p<0.01$) by two-way ANOVA followed by Tuckey's test.

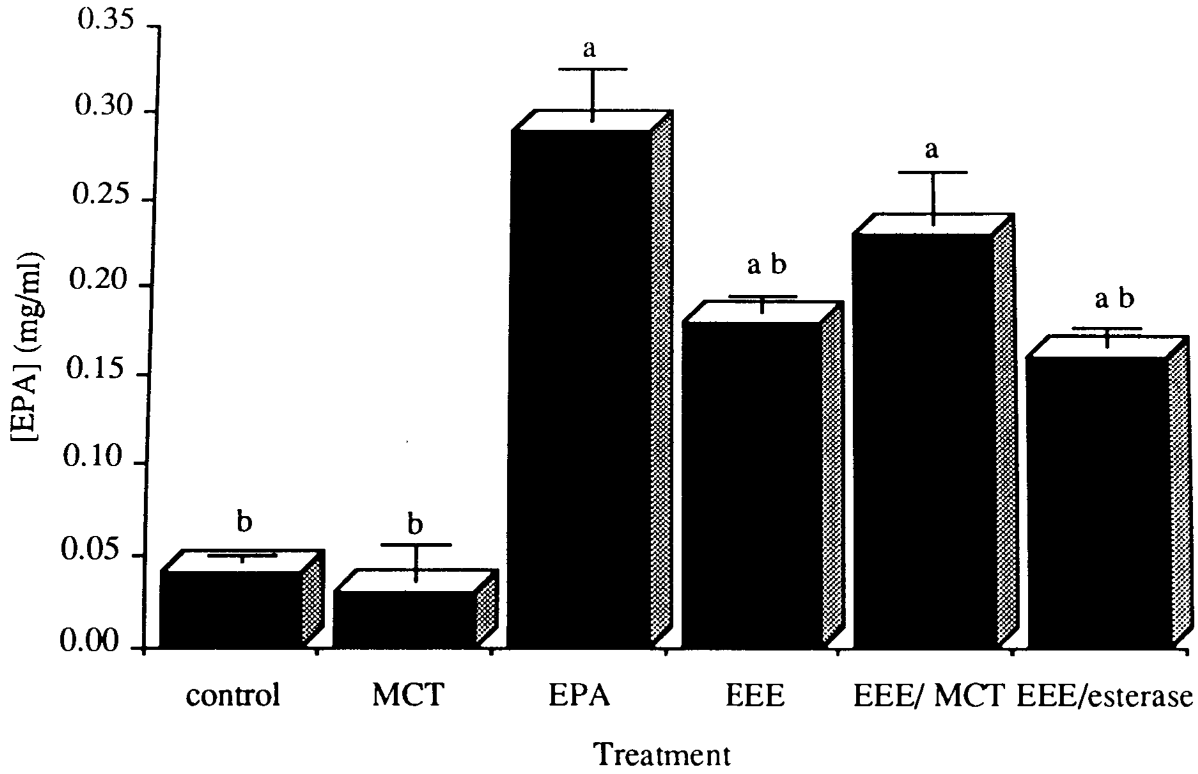


Figure 26. Tumour [EPA] after treatment with EPA (2.0g/kg), EEE (2.5g/kg), EEE (2.5g/kg) plus MCT diet and EEE pretreated with esterase.
Results are expressed as mean ± SEM for 4 tumours per group.
Significantly different from control [a] ($p<0.01$) and from EPA [b] ($p<0.01$) by one-way ANOVA followed by Tuckey's test.

4.4.3 Discussion.

In support of previous findings, neither DHA nor its ethyl ester were found to possess anti-tumour or anti-cachectic activity. Olive oil EE was likewise found to have no effect on these parameters.

The surprising lack of activity of the ethyl ester of EPA was attributed to differences in the pharmacokinetics of the uptake of EPA and its ethyl ester. Treatment with EEE compared to the free acid resulted in both lower concentrations and delayed appearance of plasma EPA; tumour levels of EPA after EEE treatment also remained below those of the EPA treated group.

These results are supported by the report that in humans the free acid of EPA was found to undergo 95% absorption from the lumen, while the triglyceride and ethyl ester showed only 68 and 20% uptake respectively (Lawson and Hughes, 1988b). Yang *et al.* (1989; 1990) have also found hydrolysis of EEE to occur at a slower rate than the triglyceride form by a factor of 4 *in vivo* and 10-15 *in vitro* depending on the relative proportions of the two esters. In both the studies of Yang *et al.* however, it was concluded that these rates were sufficient to result in a saturated micellar solution of fatty acids for absorption.

The absorption of EEE has been reported to be improved by the co-administration of a high fat diet. Lawson and Hughes (1988a) found a three-fold increase in absorption of the ethyl ester from 20 to approximately 60% when human volunteers ingested a high fat meal (44% by calories). A similar attempt to increase uptake of EEE into MAC16 tumour-bearing mice by feeding an MCT diet was not found to be successful in eliciting an anti-tumour or anti-cachectic effect from the EEE at either 2.5g/kg or 5.0g/kg. This lack of effect may be related to the observation that the MCT diet is poorly tolerated by the mice. Food intake of these animals was, although not significantly different to the control, found to be slightly depressed, and diarrhoea was common in the mice. The nature of the MCT diet means that feeding results in the coats of the mice becoming very greasy and therefore non-insulative, and it is suggested that the mice may lose a considerable amount of body heat through the skin under these conditions, resulting in an

increased metabolic rate and exacerbation of weight loss induced by the MAC16 tumour. It was also noted that the PUFA content of the MCT diet was as little as 44% of the RMB diet content, and it is possible that this alone may result in a slight decrease in tumour growth due to deprivation of sufficient quantities of LA.

It was therefore concluded that the slight tumour growth inhibition of the MCT diet groups seen in Fig.23A is due to adverse effects of the feeding of the MCT diet, and not due to increased uptake of EPA from the lumen. This is confirmed by the uptake studies, in which plasma levels of EPA in the MCT plus EEE group were found not to rise above those of the EEE group until 96 hours after the treatment was commenced. Any possible advantage to the mouse of increased plasma and tumour levels of EPA would therefore be lost, due to the rapidity of the weight loss associated with the growth of this tumour and consequent need for termination of experiments shortly after this time point.

Hydrolysis of the ethyl ester wih pig liver esterase *in vitro* before administration to MAC16 tumour-bearing mice was found to result in an 80% conversion to EPA. At the dose level administered this would have been equivalent to 2.0g/kg of the free acid. However polymerisation of the fat in the incubation buffer during the hydrolysis treatment greatly reduced the amount of both EEE and free acid available to the animal resulting in the actual administration of a much lower dose of EPA than estimated from the fatty acid analysis of the mixture. This is reflected in the inactivity of this preparation against the MAC16 tumour, and is again supported by the uptake studies which show plasma and tumour levels to be much reduced when compared to the EPA group, and to be very similar to those of the untreated EEE group.

In summary, attempts made in this study to elicit anti-tumour and anti-cachectic activity from the ethyl ester of EPA by various treatment combinations and manipulations were unsuccessful. It is suggested that for the EEE and EEE plus MCT groups, the lack of activity was due to insufficient uptake of EPA from the lumen over the time scale available in this model. The lack of success with the esterase pretreated EEE was attributed to a problem of methodology.

The MAC16 model of cachexia results in a very rapid loss of body weight over the course of approximately one week necessitating termination of experiments

after this time. Therefore given a longer time course as in the human condition, accumulation of EPA in body tissues after the administration of EEE may be sufficient to result in anti-tumour and anti-cachectic activity.

4.5 Effect of Co-administration of 5-Fluorouracil with EPA on Tumour Growth and Weight Loss of MAC16 Tumour-bearing Mice.

4.5.1 Introduction.

The anti-tumour activity of EPA has been found to be biphasic (section 4.3). The latter phase of accelerated tumour growth, occurring approximately 5 days after the initial treatment with EPA, was found to be due to a reversal of the increase in cell death seen during the first stage of EPA-induced tumour growth inhibition together with an increase in the proliferative compartment. It is therefore hypothesised that at this point the anti-tumour activity may be prolonged and enhanced by the administration of an anti-proliferative drug.

The fatty acids EPA and GLA have been shown by Plumb and Kerr (1993) to cause sensitisation to cytotoxic drugs of certain drug-resistant cell lines *in vitro*. It was suggested that this combination may be of some clinical benefit, despite the toxicities of the fatty acids and drugs being found to be additive rather than supraadditive.

The cytotoxic agent 5-fluorouracil (5-FU) is commonly used in the treatment of a wide range of tumours (Fischer and Knobf, 1989). 5-FU is thought to exert its anti-tumour activity via two possible mechanisms; firstly by inhibition of DNA synthesis by competitive inhibition of deoxythymidine monophosphate synthetase, and secondly by alterations in cell RNA as a result of the incorporation of 5-FU in the place of uracil (Stevens *et al.*, 1984).

In this study the effect of a combination treatment of EPA and 5-FU on the tumour growth and weight loss of mice bearing the MAC16 adenocarcinoma was investigated.

5-FU (80mg/kg) was administered from the fourth day of EPA treatment at intervals of 48 hours to reduce toxicity.

4.5.2 Results.

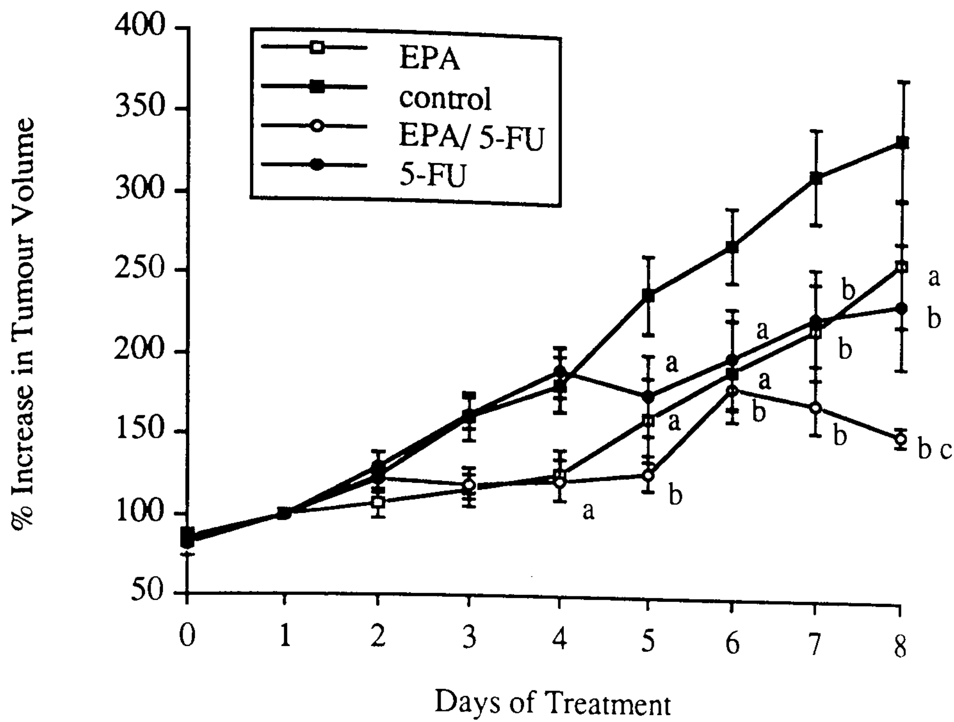
The EPA/ 5-FU combination treatment was found to reduce the tumour regrowth observed to occur after approximately 4 days in animals treated with EPA alone (Fig.27A). Treatment with 5-FU alone was also found to cause inhibition of tumour growth.

Weight loss experienced by MAC16 tumour-bearing mice was not reversed by administration of 5-FU (80mg/kg) alone (Fig.27B). However, the 5-FU treatment regime was well tolerated by the mice and weight loss was only found to be significantly increased compared to control mice on one occasion after dosing in the 5-FU alone group, indicating moderate toxicity. Administration of 5-FU at the higher concentration of 100mg/kg increased neither the anti-tumour effect nor toxicity (results not shown); administration of 5-FU at 120mg/kg has previously been found to be extremely toxic to MAC16 tumour-bearing mice (Beck, unpublished results).

No conclusions about the effect of the combination treatment on weight loss can be drawn from the data presented in Fig.27B, as the batch of EPA90 used in this series of experiments was found to cause toxicity including diarrhoea in the mice, therefore resulting in negation of the anti-cachectic effect. The toxicities of the two components did not appear however to be additive, weight loss of the combination group not being increased above control or other treatment groups.

Two treatment protocols for the combination group were tested, in group one mice received daily doses of EPA throughout the experiment, whereas in group two, EPA treatment was terminated on day 4 when 5-FU treatment was initiated. No overall advantage was conferred by either protocol over the other (Fig.28).

27A



27B

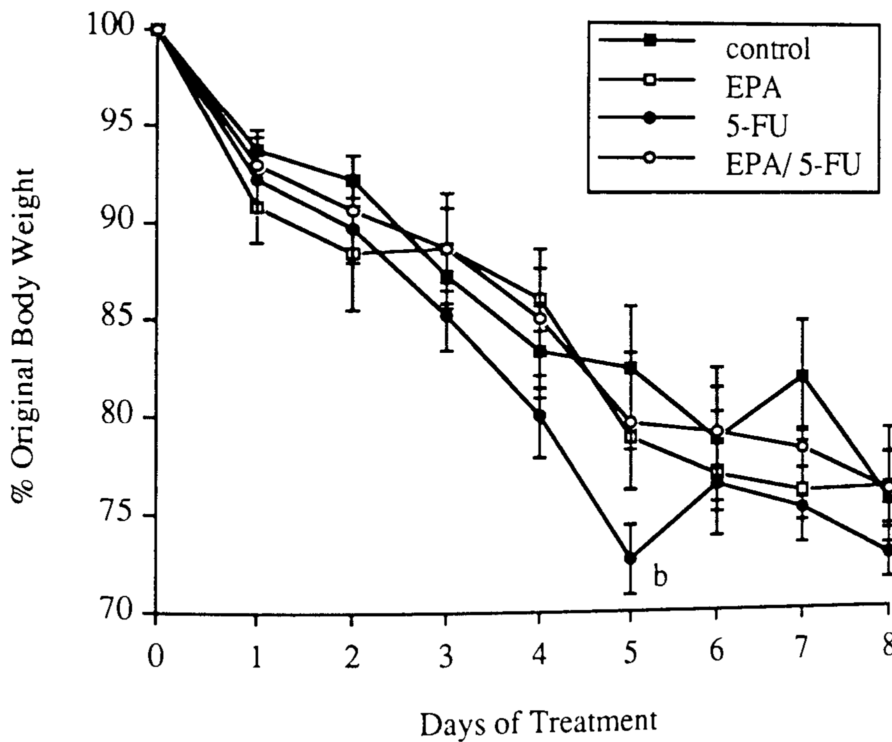


Figure 27. Effect of EPA (2.25g/kg), 5-FU (80.0mg/kg) and EPA plus 5-FU on tumour growth (A) and weight loss (B) of MAC16 tumour-bearing mice. 5-FU was administered 48 hourly from day 4.
Results are expressed as mean ± SEM for 6 animals per group.
Significantly different from control [a]($p<0.05$), [b]($p<0.01$) and from all other groups [c]($p<0.01$) by two-way ANOVA followed by Tuckey's test.

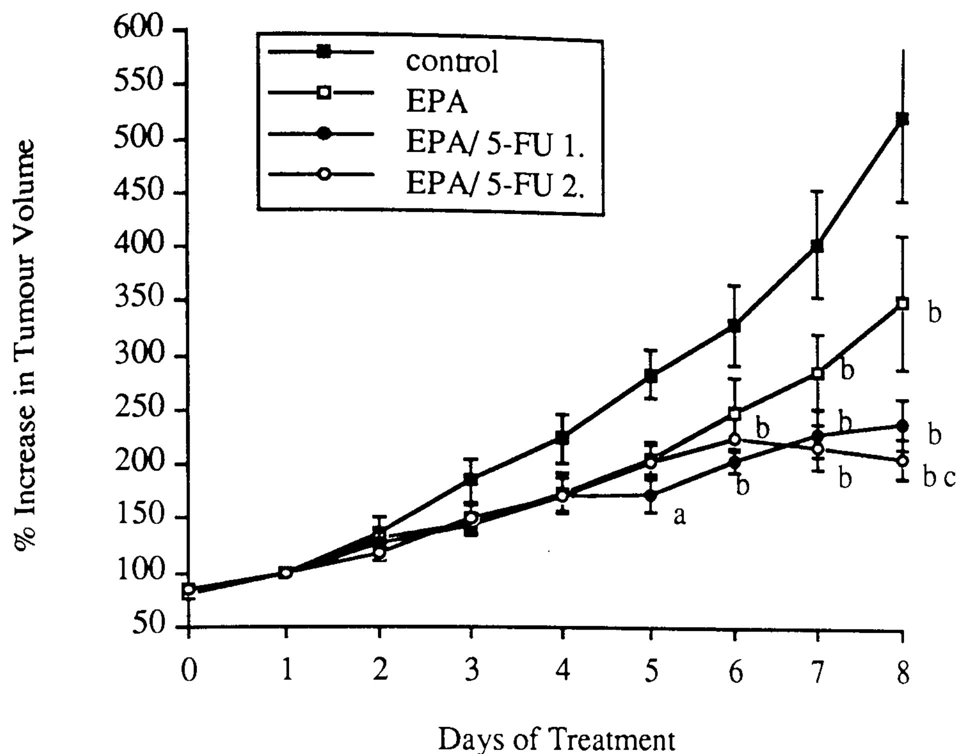


Figure 28. Effect of EPA (2.25g/kg) and EPA plus 5-FU (80.0mg/kg) on tumour growth of MAC16 tumour-bearing mice. Treatment EPA/ 5-FU1. indicates continuous administration of EPA during 5-FU treatment, while treatment EPA/ 5-FU2. indicates cessation of EPA administration on day 4 of treatment. 5-FU was administered 48 hourly from day 4.

Results are expressed as mean ± SEM for 6 animals per group.

Significantly different from control [a](p<0.05), [b](p<0.01) and from EPA [c](p<0.01) by two-way ANOVA followed by Tuckey's test.

4.5.3 Discussion.

The anti-tumour effect of EPA was found to be enhanced by treatment with 5-FU, causing slight regression of the tumours after day 6 in two out of three groups, and a delay in the regrowth of the tumour in two of the three cases shown in Figs.27 and 28. Administration of 5-FU alone was also found to result in substantial tumour growth inhibition.

It was concluded that for the combination protocols employed in this study, the prolonged anti-tumour activity was due to additive effects of the two treatments and not to synergy.

Animals receiving 5-FU either alone or in combination with EPA experienced moderate toxicity including diarrhoea and general malaise, although weight loss was not significantly increased above the control group. The weight loss associated with the EPA90 treatment was found to be specific to the batch used in this experiment, and remains unexplained. Toxicity may have resulted due to solvent contamination of the EPA, or due to the co-purification of a toxic factor normally in low concentrations in the less pure EPA80 preparation.

These results indicate a potential for the combination of EPA with an anti-proliferative drug, although further investigation is required for the development of a suitable protocol.

4.6 Effect of Cyclooxygenase and Lipoxygenase Inhibitors on the Tumour Growth and Weight Loss of MAC16 Tumour-bearing Mice.

4.6.1 Introduction.

It is widely recognised that LA is capable of stimulating the growth of neoplastic cells both *in vitro* and *in vivo* (sections 1.4 and 4.2). The mechanism by which this stimulation occurs is as yet unclear. LA is chiefly metabolised to AA, the major precursor of the eicosanoids. There are three main pathways of eicosanoid biosynthesis from AA, the cyclooxygenase pathway in which AA is converted to prostaglandins (PG) and thromboxanes (TX); the lipoxygenase pathway leading to the formation of hydroperoxyeicosatetraenoic acids (HpETEs) and leukotrienes (LT); and the epoxygenase pathway in which AA is metabolised to *cis*-epoxy-eicosatrienoic acids (EpETEs) and hydroxyeicosatetraenoic acids (HETEs). Therefore a role for one or more of these metabolites in tumour growth is indicated.

A number of studies have presented evidence implicating the cyclooxygenase pathway in neoplastic growth, Horrobin (1980) has commented that a characteristic of cancer cells is their overproduction of the 2 series of prostaglandins. These PGs, particularly PGE_2 have also been found to be decreased in tumours in which growth has been inhibited by the administration of marine oil (Karmali *et al.*, 1984; 1987). The inhibitory effect of dietary supplementation with n-3 fatty acids on prostanoid formation (particularly of PGE_2 and TXB_2) has been confirmed in a range of other studies both in healthy human volunteers (Ferretti and Flanagan, 1990; Weber *et al.*, 1986) and in non tumour-bearing animals (Terano *et al.*, 1986).

Growth of the M5075 ovarian reticulosarcoma has been found to be associated with increases of TXB_2 and PGE_2 (Chiabrando *et al.*, 1987). This study also found that total inhibition of TXB_2 synthesis resulted in increased synthesis of PGs and increased tumour growth, indicating that while TXB_2 may not be a requirement for tumour growth, PGs may play a role in tumour growth stimulation.

Administration of indomethacin, a cyclooxygenase inhibitor has been found to inhibit or reduce tumour growth in patients with head and neck carcinomas (Panje, 1981) and also to inhibit tumour growth and improve the nutritional status of cachectic tumour-bearing mice (Gelin *et al.*, 1991).

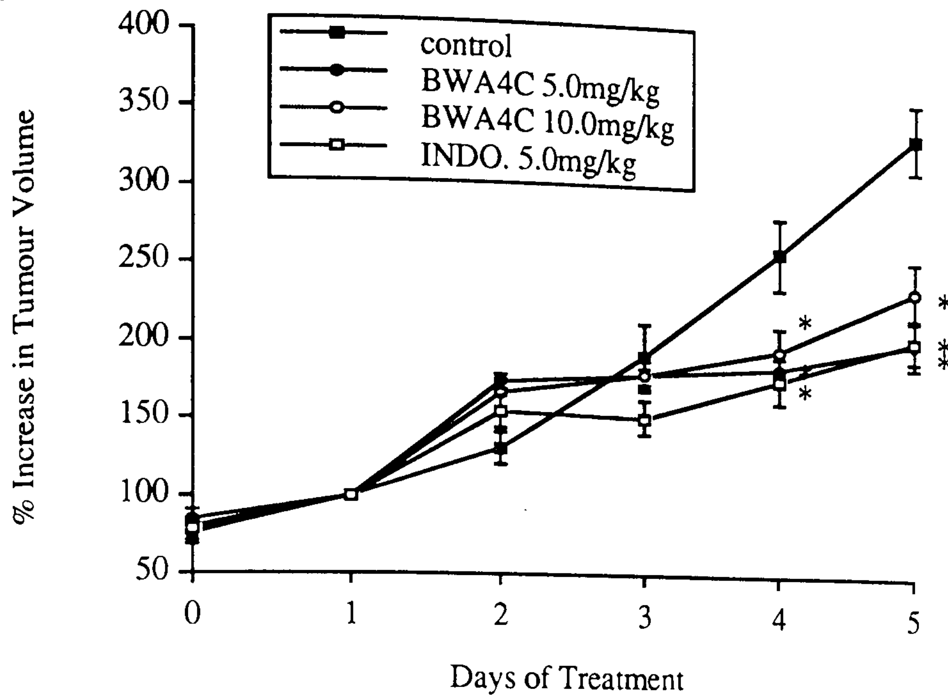
In contrast, Buckman *et al.* (1991) have reported increased growth of a metastatic mouse mammary tumour cell line *in vitro* in response to cyclooxygenase inhibition, while lipoxygenase inhibition resulted in a dose related decrease in cell growth. Lipoxygenase products were also implicated in the LA stimulation of growth of this cell line. Studies by Abou-El-Ala *et al.* (1989) found inhibition of growth of DMBA induced tumours *in vivo* by dietary supplementation with menhaden oil to be associated with a decrease in LTB_4 and PG synthesis. Administration of indomethacin was found to result in inhibition of cyclooxygenase products without tumour growth inhibition, suggesting that the inhibitory effect of fish oil was due to decreased levels of LTB_4. A role for LTB_4 in the pathogenesis of head and neck cancer has also been proposed (El-Hakim *et al.*, 1989).

The aim of the study presented here is to investigate the effects of the cyclooxygenase inhibitors indomethacin and 5,8,11,14-eicosatetraynoic acid (ETYA) and of the lipoxygenase inhibitors BW A4C and BW B70C on the growth and cachexia of the MAC16 model.

4.6.2 Results.

Data presented in Figure 29 indicate that both cyclooxygenase and lipoxygenase inhibitors (indomethacin and BW A4C respectively) are capable of causing tumour growth inhibition in the MAC16 model of cachexia when administered as a suspension in 1% methylcellulose. Weight loss appeared to be slightly decreased in the BW A4C (5.0mg/kg) group, although this was not significant.

29A



29B

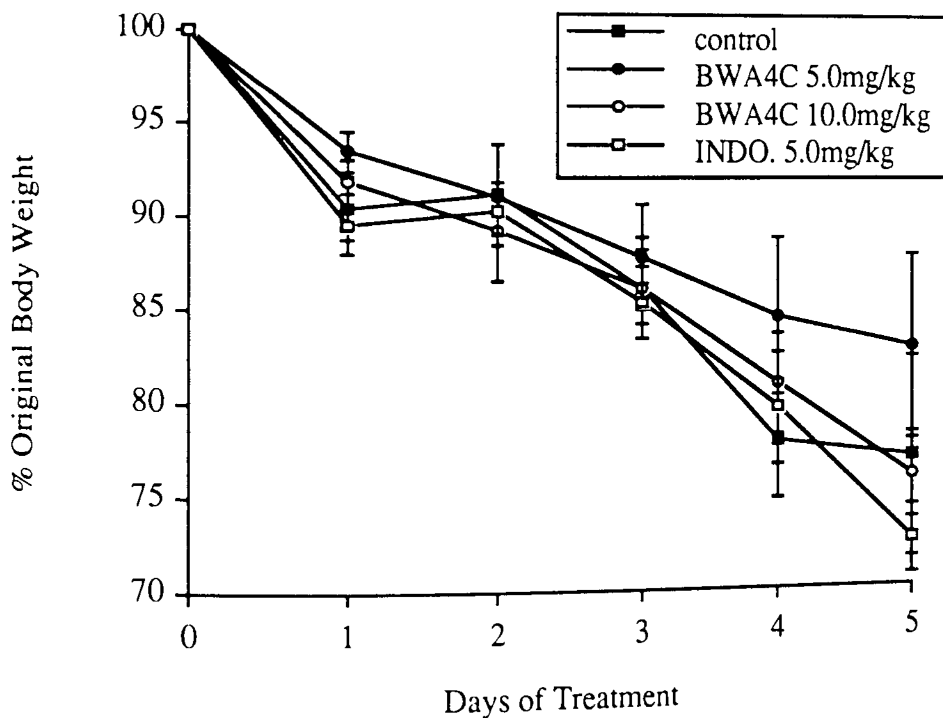


Figure 29. Effect of BW A4C (5.0, 10.0mg/kg) and indomethacin (5.0mg/kg) in a 1% methylcellulose suspension on tumour growth (A) and cachexia (B) of the MAC16 model.
Results are expressed as mean ± SEM for 6 animals per group.
* significantly different from control ($p<0.01$) by two-way ANOVA followed by Tuckey's test.

Eicosatetraynoic acid (ETYA) was found to be toxic at both 5.0 and 10.0mg/kg, while having no anti-tumour effect at these concentrations (results not shown). At the lower concentration of 2.5mg/kg indomethacin was also found to be inactive (results not shown).

The anti-tumour effect of the BW A4C compound was found to be reproduceable following administration as a suspension in 0.25% carboxymethylcellulose (Fig.30). This effect was not however found to be dose dependent, and again no anti-cachectic effect was observed. The lipoxygenase inhibitor BW B70C was found not to have either anti-tumour or anti-cachectic properties when administered as a suspension in 0.25% carboxymethylcellulose (Fig.31).

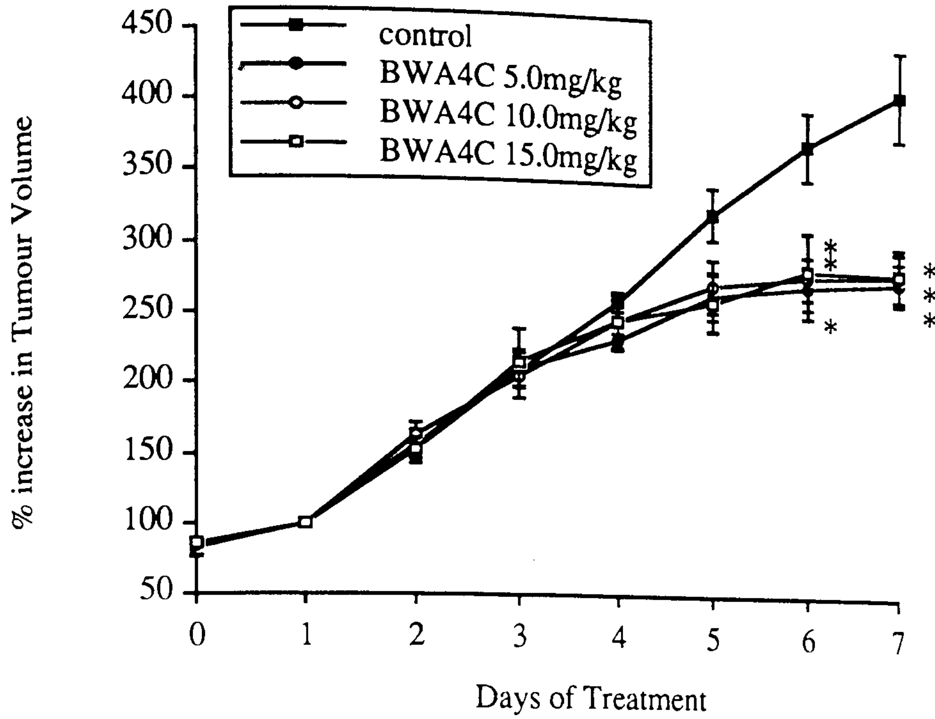
The anti-tumour effect obtained with indomethacin in 1% methylcellulose was found to be less pronounced when administered in 0.25% carboxymethylcellulose, treatment causing only a slight delay in tumour growth which was found not to be significant (Fig.32). Weight loss was found not to be significantly different from the control group, although slightly greater in the treatment group on days 5 and 6.

Suspension of the compounds in either 1% methylcellulose or 0.25% carboxymethylcellulose was found not to be a satisfactory method of administration, the viscosity of the solutions causing difficulties in administration. Compounds were found to be soluble in DMSO, and a solution of 10% DMSO was better tolerated by the animals.

When administered as solution in 10% DMSO however, the anti-tumour activity of compound BW A4C was lost at all concentrations tested (1.0, 2.5, 5.0 and 10.0mg/kg) (Fig.33), (20.0 and 40.0mg/kg) (results not shown). As in previous experiments, no anti-cachectic activity was apparent.

The cyclooxygenase inhibitor indomethacin (in 10% DMSO) was found to have pronounced anti-tumour activity at 5.0 and 10.0mg/kg; weight loss was slightly decreased by the 5.0mg/kg group (not significant) while the higher concentration caused toxicity, resulting in the termination of that treatment group on day 5 (Fig.34)

30A



30B

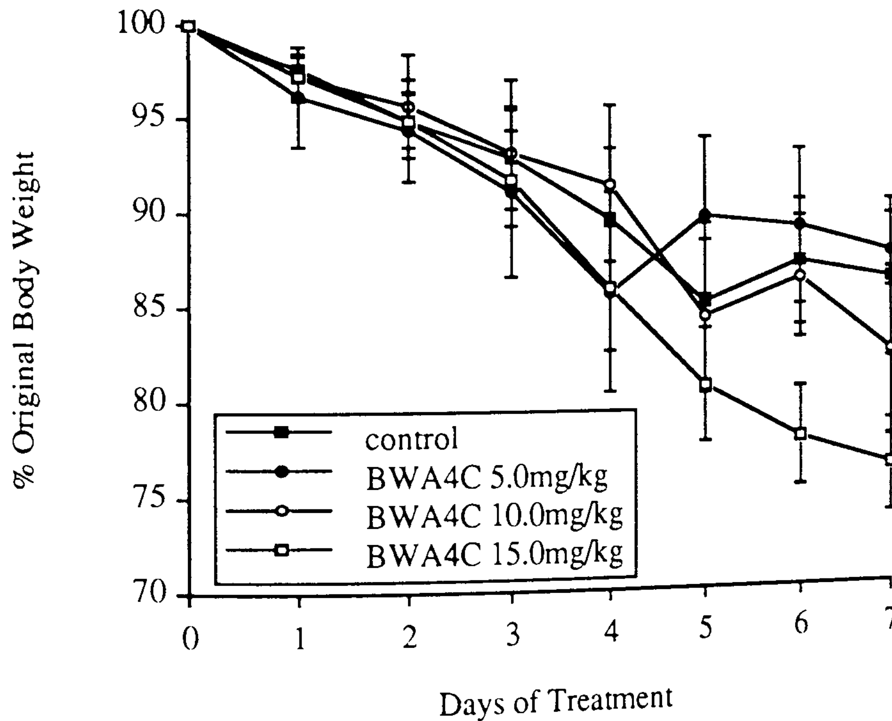
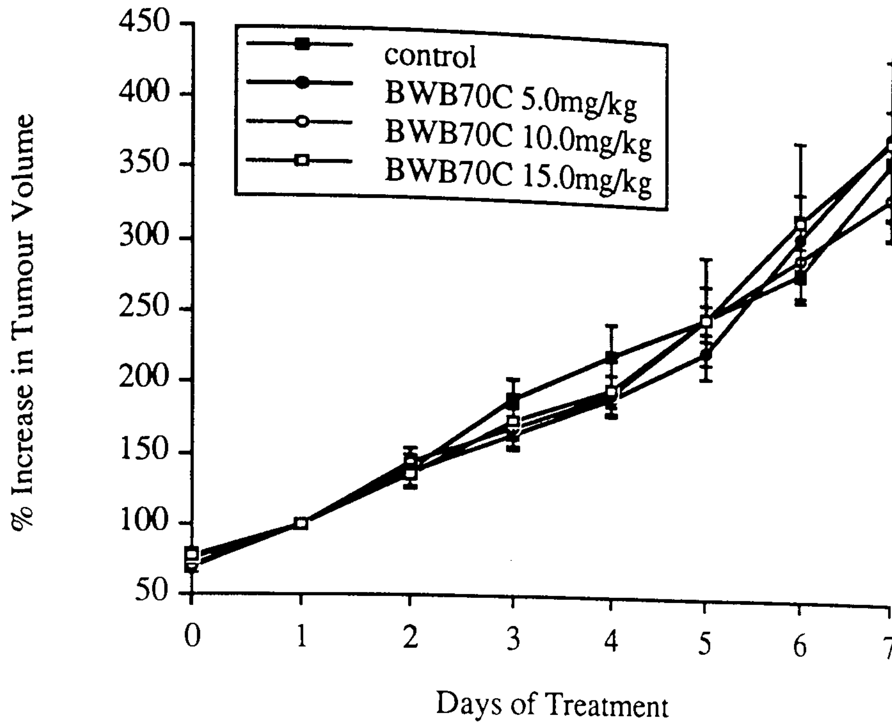


Figure 30. Effect of the lipoxygenase inhibitor BW A4C (in 0.25% carboxymethylcellulose) on tumour growth (A) and weight loss (B) of the MAC16 model.
Results are expressed as mean ± SEM for 6 animals per group.
* significantly different from control ($p<0.01$) by two-way ANOVA followed by Tuckey's test.

31A



31B

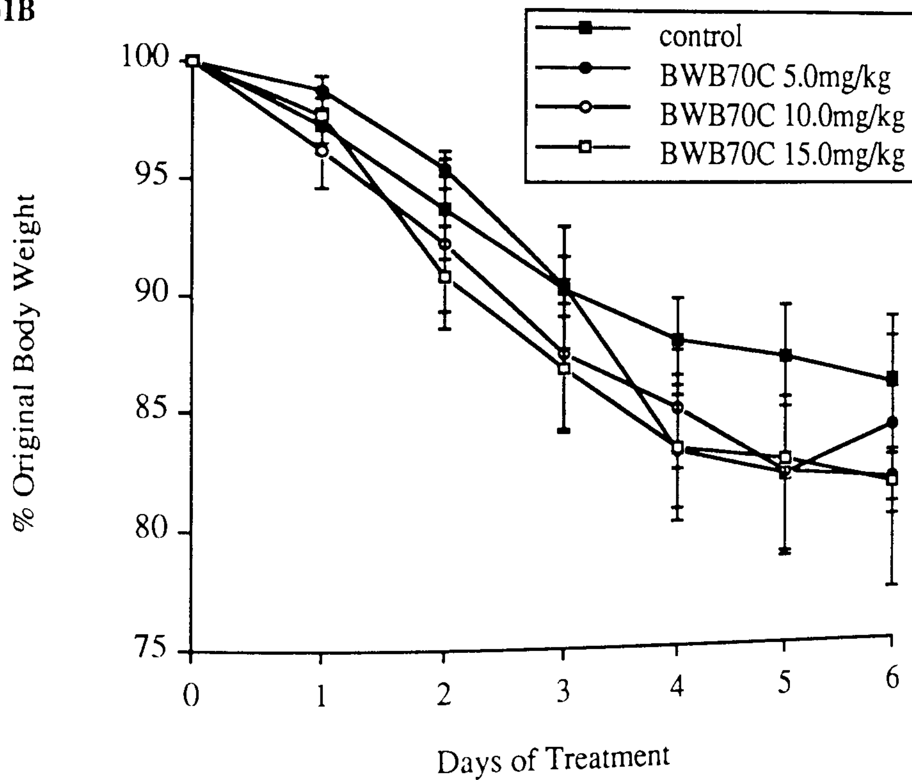
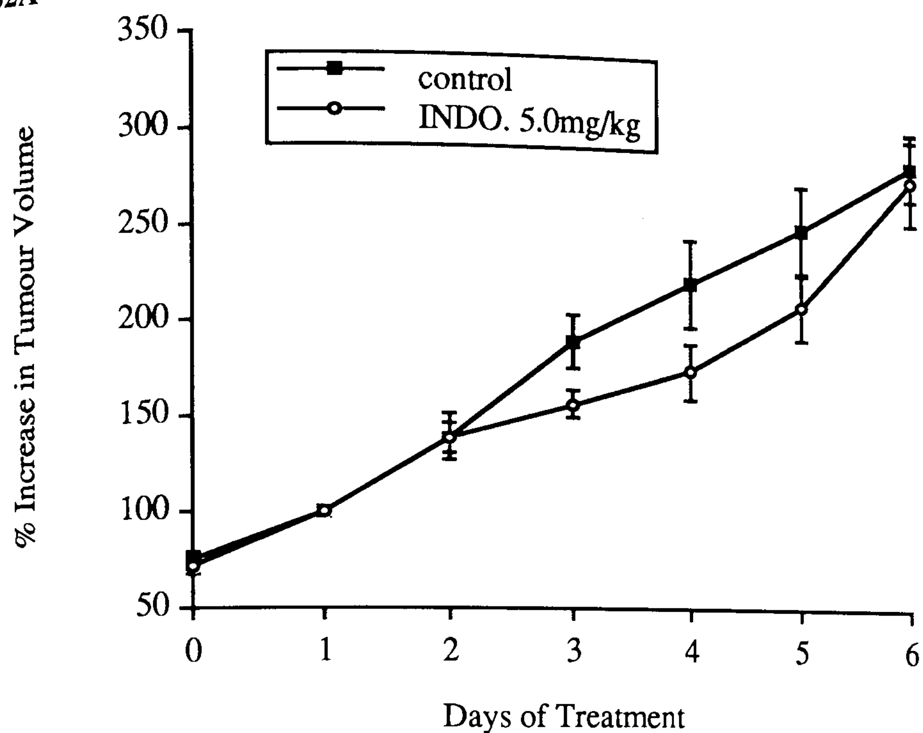


Figure 31. Effect of the lipoxygenase inhibitor BW B70C (in 0.25% carboxymethylcellulose) on tumour growth (A) and weight loss (B) in the MAC16 model.
Results are expressed as mean ± SEM for 6 animals per group.

32A



32B

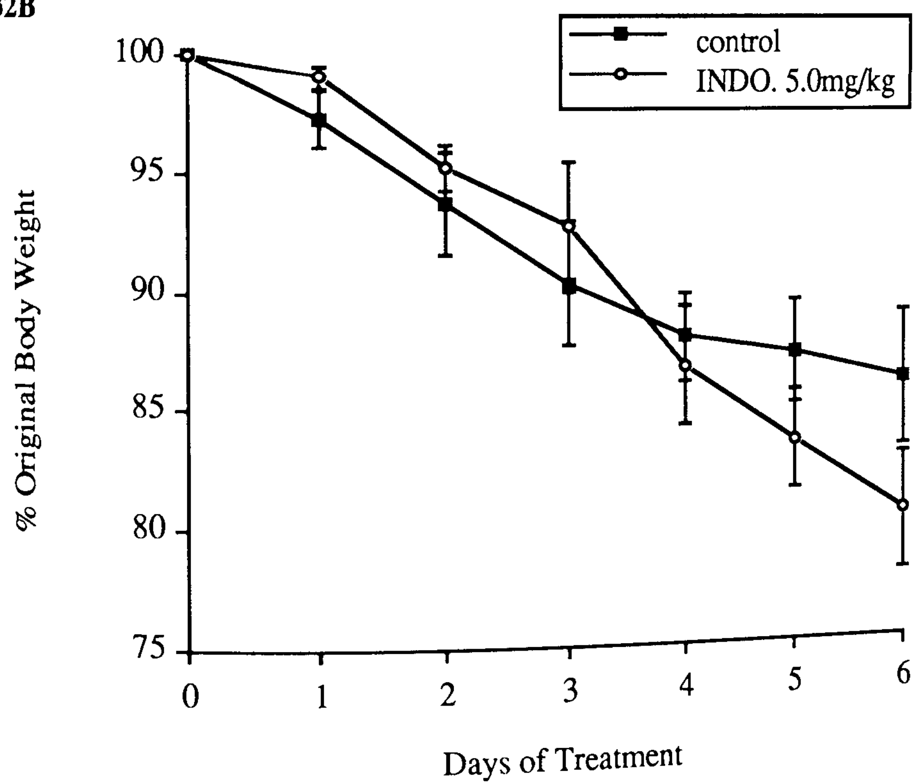


Figure 32. Effect of the cyclooxygenase inhibitor indomethacin (5.0mg/kg in a suspension of 0.25% carboxymethylcellulose) on tumour growth (A) and weight loss (B) of the MAC16 model. Results are expressed as mean ± SEM for 6 animals per group.

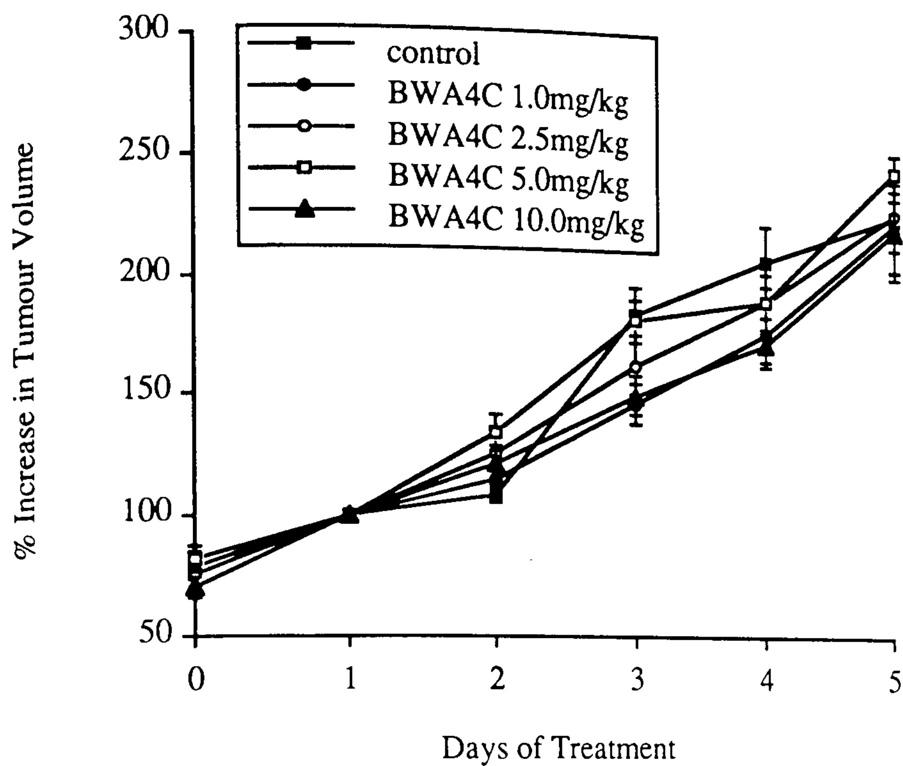
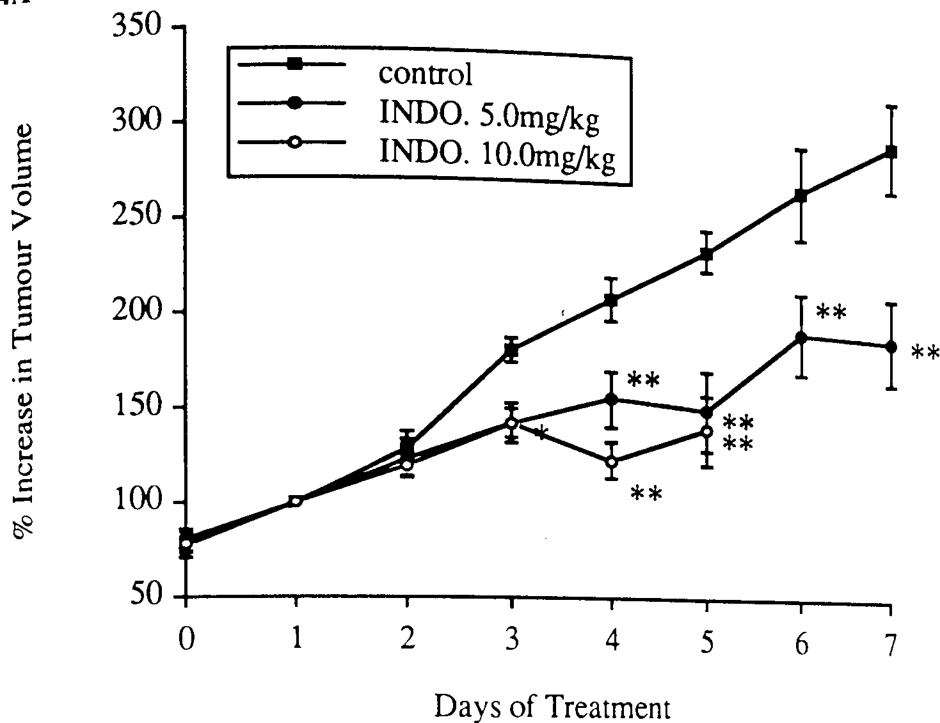


Figure 33. Effect of BW A4C (in 10% DMSO) on tumour growth of MAC16 tumour-bearing mice.
Results are expressed as mean ± SEM for 6 animals per group.

34A



34B

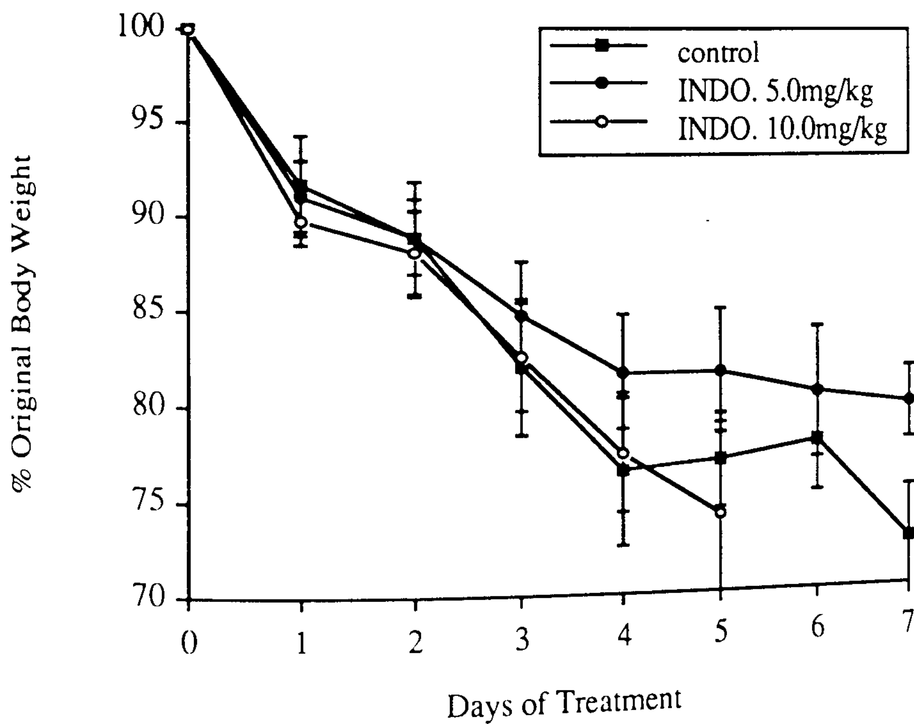
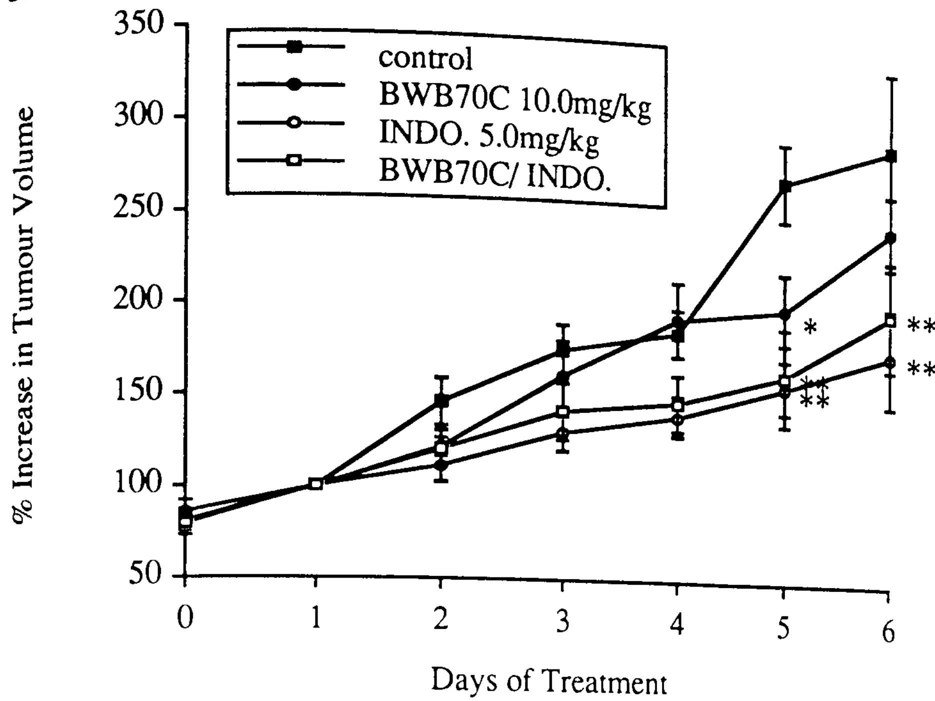


Figure 34. Effect of indomethacin (in 10% DMSO) on tumour growth (A) and weight loss (B) of the MAC16 model.
Results are expressed as mean ± SEM for 6 animals per group.
Significantly different from control *($p<0.05$) **($p<0.01$) by two-way ANOVA followed by Tuckey's test.

The lipoxygenase inhibitor BW B70C (10.0mg/kg in 10% DMSO) was found to cause a slight delay in tumour growth after 4 days of treatment. Combination of BW B70C with the cyclooxygenase inhibitor indomethacin (5.0mg/kg in 10% DMSO) was not found to increase the anti-tumour effect achieved by administration of indomethacin alone (Fig.35). No reduction in weight loss occurred after any treatment.

35A



35B

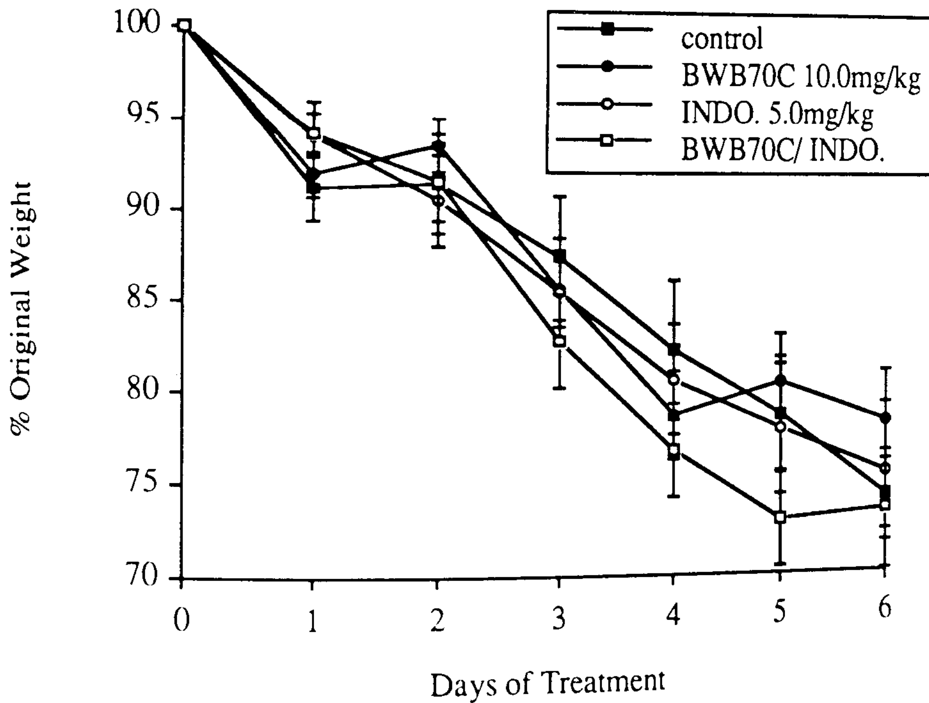


Figure 35. Effect of the combination of the lipoxygenase inhibitor BW B70C (10.0mg/kg in 10% DMSO) and the cyclooxygenase inhibitor indomethacin (5.0mg/kg in 10% DMSO) on tumour growth (A) and weight loss (B) of the MAC16 model.
Results are expressed as mean ± SEM for 6 animals per group.
Significantly different from control *($p<0.05$) **($p<0.01$) by two-way ANOVA followed by Tuckey's test.

4.6.3 Discussion.

The compound BW A4C has been found to be a potent inhibitor of 5-lipoxygenase activity, leading to inhibition of the synthesis of LTB_4, 5-HETE and TXB_2 *in vitro* (Tateson *et al*, 1988).

In this study, BW A4C was administered to animals as a suspension in either 1% methylcellulose or 0.25% carboxymethylcellulose, and as a solution in 10% DMSO. When administered as a suspension in either of the two cellulose solutions, BW A4C was found to result in inhibition of tumour growth, although this effect was not found to be dose related (Figs.30 and 31). The compound did not however exert any anti-cachectic effect. This result was unusual, as inhibition of tumour growth would be expected to be accompanied by a decrease in the weight loss experienced by the animals. This may be explained by toxicity of the compound itself masking any reduction in weight loss which may have occurred as a result of tumour growth inhibition.

In contrast, when administered in 10% DMSO, BW A4C was found not to exert anti-tumour activity. It is suggested that this was due to increased rates of plasma uptake and clearance of the drug when presented in this form, resulting in inadequate exposure of tumour cells to the compound. BW A4C has been found to be a potent inhibitor of growth of the MAC16 cell line *in vitro* (Hussey, unpublished results).

A second lipoxygenase inhibitor, compound BW B70C was found to be inactive when administered as a suspension in 0.25% carboxymethylcellulose, but caused slight tumour growth inhibition when administered in 10% DMSO. As found for BW A4C, treatment with BW B70C did not result in a reduction in the weight loss associated with tumour growth.

The inibition of MAC16 tumour growth *in vivo* by the cyclooxygenase inhibitor indomethacin confirms previous results obtained by Gelin *et al.* (1991) with mice bearing a methylcholanthracene induced sarcoma. MAC16 tumour growth inhibition in this study was observed in groups in which indomethacin was administered as a suspension in 1% methylcellulose or as a solution in 10% DMSO.

he lack of effect obtained with the carboxymethylcellulose suspension is
ıexplained. As with the lipoxygenase inhibitors, no reversal of weight loss was
ɔserved, this was again attributed to toxicity of the treatment.

The effect of combining inhibitors of both these pathways (BW B70C plus
ıdomethacin) was found not to be synergistic, tumour growth inhibition being very
imilar to that achieved by the administration of indomethacin alone. It is possible
owever that this was due to the poor nature of tumour growth inhibition obtained
/ith BW B70C, and a greater effect may be seen using a more potent inhibitor.

In summary, both inhibitors of the lipoxygenase, and cyclooxygenase
ıathways were found to exert anti-tumour activity against the MAC16 tumour *in
'ivo*. Inhibition of neither pathway however resulted in reduction or reversal of
veight loss experienced by the mice, it is suggested that this may be due to toxicity
esulting from the treatment itself. These results support the *in vitro* findings of Lee
ınd Ip (1992) and Rose and Connolly (1991) in which inhibition of a rat mammary
umour line, and a human prostatic cancer cell line respectively, could be achieved
ɔy administration of inhibitors of either the cyclooxygenase or lipoxygenase
pathways.

It is concluded therefore, that products of both cyclooxygenase and
lipoxygenase pathways of AA metabolism are important in the regulation of growth
of the MAC16 tumour *in vivo*, and that any disruption of this balance may lead to
inhibition of tumour growth.

SECTION 5.0 CONCLUSIONS.

5.0 CONCLUSIONS.

Results presented in section 4.1 show the breakdown of adipose tissue, and mobilisation of fatty acids to occur in a non specific manner in response to the growth of the MAC16 tumour. Liver size in MAC16 tumour-bearing animals was also found to decrease with increasing weight loss, and results indicated an increased capacity of the liver to concentrate fatty acids in response to cachexia. Total plasma fatty acids were found to increase with increasing weight loss; this was found to be mainly due to small increases in all individual fatty acids, although the increase was found to be particularly marked in AA concentration. It was also observed that at low levels of weight loss both liver and plasma fatty acid concentrations of MAC16 tumour-bearing mice were lower than average levels of non tumour-bearing control mice. The plasma ratio of SA:OA was found to increase with both weight loss and tumour volume. The total fatty acid content of the tumour was found to increase with tumour size as would be expected, although the specific concentration of the fatty acids was found to decrease. Studies with the serum of human pancreatic cancer patients showed a peak in AA concentration at 10 to 15% weight loss, thus supporting results obtained with the animal model for an increase in plasma AA with weight loss. In contrast to results obtained with the MAC16 model however, the SA:OA ratio was found to decrease with weight loss in these patients.

From these studies, it is concluded therefore, that initial growth of the tumour is accompanied by its non-specific sequestration of fatty acids from the plasma and liver leading to a drop in the fatty acid content of these tissues compared with values obtained for non tumour-bearing animals. This phase is rapidly followed by the entrance of the tumour into its exponential phase of growth, and the onset of cachexia. In response to production of the lipolytic factor, results suggest that fatty acids were mobilised from adipose stores in a non-specific manner, leading to an increase in liver and circulatory fatty acid levels as weight loss increased. The tumour was not found to preferentially concentrate a particular fatty acid during its growth. The non-specificity of the results obtained in this study may

represent the inaccuracies inherent in the measurement of total fatty acid content, rather than the determination of individual values for the phospholipid, triglyceride and cholesterol compartments. Results from both mouse and human studies may suggest a role for circulatory AA in the develpoment of cachexia.

Sections 4.2 and 4.3 investigated the mechanism of the anti-tumour and anti-cachectic activity of EPA, and also the reversal of the anti-tumour effect by other PUFAs. EPA was found to cause a decrease in both plasma and tumour levels of AA in animals receiving treatment. It was determined however, that this was not the mode of action of EPA, as treatment with the n-3 fatty acid DHA, which is inactive against the MAC16 tumour *in vivo*, was also found to cause these changes in fatty acid composition. Arachidonic acid levels were also depressed in tumours of mice treated with the tumour growth promoter, linoleic acid, thus bringing into question the role of arachidonic acid in tumour growth stimulation and the development of cancer cachexia.

The PUFAs LA and OA both reversed the anti-tumour, but not the anti-cachectic effect of EPA, and this reversal of the anti-tumour effect was found not to be due to decreased incorporation of EPA into the tumour. This suggests that separate mechanisms exist for the anti-tumour and anti-cachectic activities of EPA, and that the anti-tumour effect of EPA is indirect. Triglycerides of LA and OA in the form of corn oil were found not to reverse the anti-tumour activity of EPA, this was attributed to the lack of an increase in the concentration of these PUFAs in either plasma or tumour following dosing.

An investigation of the cell kinetics of EPA tumour growth inhibition showed an increase in the rate of cell loss from 38 to 71% in response to treatment with EPA, indicating that EPA acts by increasing cell death rather than by inhibiting cell proliferation. Studies performed by Drs M. Bibby and J. Double (Bradford University) showed increased areas of necrosis in MAC16 tumours in response to EPA treatment suggesting that increased cell death is not mediated via induction of apoptosis in this model. The finding that EPA acts via the increasing of cell loss from the tumour rather than by inhibition of cell proliferaton has important implications for its clinical application, as the devastating side effects associated with conventional chemotherapeutic treatments comes generally from their toxicity

to areas of rapidly dividing cells other than the tumour, such as the gastrointestinal mucosa.

The anti-tumour effect of EPA was found to be biphasic, the latter stage of which was characterised by an increased rate of tumour growth. This exponential growth was found to be associated with a decrease in cell loss compared with stage 1 of EPA treatment, and a decrease in the potential doubling time compared with that of both EPA stage 1 tumours and untreated tumours. Reversal of EPA induced tumour growth inhibition by the co-administration of LA was also found to be attributable to a decrease in cell loss from 71 to 45%. This lead to the proposal that LA or one of its metabolites is essential for the maintenance of MAC16 tumour cell viability *in vivo*.

This is supported by the finding that administration of LA was also capable of stimulating the growth of the MAC16 tumour *in vivo*. The degree of this stimulation was found however to be dependent on the original growth rate of the tumour prior to treatment with the PUFA. For slow growing tumours, which might be expected to have a low rate of proliferation and a high rate of cell loss, LA appeared to be rate limiting, supplementation resulting in stimulation of tumour growth above the rate of the control. In contrast, supplementation of faster growing tumours (rapid proliferation, lower cell loss) with LA resulted in no increase in tumour growth, suggesting that in these tumours LA was not limiting.

It may be hypothesised therefore, that stimulation of the growth of slow growing MAC16 tumours may occur as a result of a decrease in the rate of cell loss, instead of or as well as by an increase in cell proliferation. The indirect action of the tumour growth inhibition by EPA leading to an increase in cell loss may therefore involve inhibition of the lipolytic factor at the site of the adipocyte, thus depriving the tumour of LA. Stimulation by LA of the growth of the slower growing MAC26 tumour, which is histologically related to the MAC16 although growing without the expression of cachexia, was not found to be due to a decrease in cell loss however, but to be due to an increase in the rate of proliferation. This suggests the potential for a dual effect of LA, both in the stimulation of proliferation and inhibition of cell loss in tumours.

The ethyl ester of EPA is more readily available than the free acid in a pure preparation, and therefore in section 4.4, the effect of the ethyl ester of EPA on tumour growth and weight loss of tumour-bearing mice was assessed. Surprisingly, EEE was found to be inactive against the tumour, as were the ethyl esters of DHA and olive oil. This lack of effect of EEE was found to be due to a lag in the appearance of the free fatty acid in both plasma and tumour of treated animals. Uptake was increased by feeding with a high fat diet, although levels were still found not to be sufficient to produce anti-tumour or anti-cachectic activity. The diet was also found to be poorly tolerated by the mice. An attempt to increase uptake of the free acid by hydrolysing EEE prior to dosing the animals was also unsuccessful, as although the hydrolysis resulted in 80% conversion of EEE to EPA free acid, much of the fatty acid became polymerised, and therefore the EPA available to the mice was again insufficient to result in an anti-tumour or anti-cachectic effect. The longer time period available for the treatment of human patients however, may allow for the use of the ethyl ester as an anti-cachectic and anti-tumour therapy.

As previously stated, the phase of exponential growth occurring after 4 to 5 days of treatment with EPA was found to be due to a decrease in cell loss, and an increase in the proliferative compartment of the tumour. It was hypothesised therefore that at this time, the MAC16 tumour may be particularly sensitive to treatment with an anti-proliferative drug. In section 4.5 the effect of treatment with 5-FU on alternate days from day 4 of EPA treatment was reported. 5-FU was found to possess anti-tumour activity against the MAC16 when administered alone, and when in combination with EPA reduced the second phase of tumour regrowth in EPA treated animals. The effect under this protocol was not as dramatic as might have been hoped however, but indicated a potential for the combination of EPA with an anti-proliferative drug as an anti-cancer treatment.

The stimulation of tumour growth, and reversal of EPA-induced tumour growth inhibition by LA combined with the observation of a possible relationship between circulating AA and cachexia (section 4.1), leads to the implication of the metabolites of AA in tumour growth and the progression of cachexia. The effect of the cyclooxygenase inhibitor indomethacin, and lipoxygenase inhibitors BW A4C

and BW B70C on growth of the MAC16 were investigated and results presented in section 4.6. Inhibition of both of these pathways of AA metabolism resulted in inhibition of growth of the MAC16 tumour, while no effect was seen on the associated weight loss. Tumour growth inhibition was not found to be dose related for any compound tested, and the lack of reversal of weight loss was attributed to toxicity of the treatments themselves. It was concluded from this study, that a balance of prostaglandins, leukotrienes and related products is required for growth of the MAC16, and disturbance of this balance by inhibition of one of these pathways of AA metabolism may result in tumour growth inhibition. EPA is a substrate for both cyclooxygenase and lipoxygenase pathways, resulting in competitive inhibition of AA metabolism, this may represent a further mode of anti-tumour activity of EPA, which may be reversed by the administration of LA.

This study has confirmed a role for LA or one of its metabolites in tumour growth and the development of cachexia, although the involvement of arachidonic acid in this process is unclear. Current knowledge concerning the anti-tumour and anti-cachectic effect of EPA has also been furthered.

SECTION 6.0 REFERENCES.

6.0 REFERENCES.

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SECTION 7.0 APPENDICES.

Appendix 1. ABBREVIATIONS.

AA	Arachidonic acid (all-*cis*-5,8,11,14-eicosatetraenoic acid).
ANOVA	Analysis of variance.
BCAAs	Branched chain amino acids.
CO	Corn oil.
CoA	Coenzyme A.
DEE	DHA ethyl ester.
DHA	All-*cis*-4,7,10,13,16,19-docosahexaenoic acid.
DMSO	Dimethyl sulphoxide.
EE	Ethyl ester.
EEE	EPA ethyl ester.
EFA	Essential fatty acid.
EN	Enteral nutrition.
EPA	All-*cis*-5,8,11,14,17-eicosapentaenoic acid.
EpETEs	*Cis*-epoxy-eicosatrienoic acids.
ETYA	5,8,11,14-eicosatetraynoic acid.
FLM	Fraction labelled mitoses.
5-FU	5-Fluorouracil.
FTPN	High fat TPN.
GC	Gas chromatogram.
GLA	γ-Linolenic acid (all-*cis*-6,9,12-octadecatrienoic acid).
HETEs	Hydroxyeicosatetraenoic acids.
HpETEs	Hydroperoxyeicosatetraenoic acids.
^{3}H-Tdr	[^{3}H]-deoxythymidine.
IGF	Insulin-like growth factor.
IL-6	Interleukin-6.
^{125}I-Udr	[^{125}I]-5-iodo-2'-deoxyuridine.

INDO.	Indomethacin.
LA	Linoleic acid (all-*cis*-9,12-octadecadienoic acid).
LDL	Low density lipoproteins.
LMF	Lipid mobilising factor.
LT	Leukotrienes.
MA	Margaric acid.
MAC	Murine colon adenocarcinoma.
MCT	Medium chain triglyceride.
MeMA	Methyl margaric acid.
NTB	Non tumour-bearing.
OA	Oleic acid (*cis*-9-octadecenoic acid).
OEE	Olive oil ethyl ester.
PA	Palmitic acid (hexadecanoic acid).
PG	Prostaglandin.
POA	Palmitoleic acid (*cis*-9-hexadecenoic acid).
PUFA	Polyunsaturated fatty acid.
RMB	Rat and mouse breeding diet.
SA	Stearic acid (octadecanoic acid).
SEM	Standard error of the mean.
TB	Tumour-bearing.
T_D	Tumour doubling time.
TNF	Tumour necrosis factor/ cachectin.
T_P	Potential tumour doubling time
TPN	Total parenteral nutrition.
TX	Thromboxane.
ϕ	Cell loss factor.

Appendix 2. PUBLICATIONS.

Hudson, E.A., Beck, S.A. and Tisdale, M.J. (1993). Kinetics of the inhibition of tumour growth in mice by eicosapentaenoic acid-reversal by linoleic acid. *Biochemical Pharmacology* **45,** (11) 2189-2194.

Abstracts.

Hudson, E.A., Hussey, H.J., Beck, S.A., Wynter, M.P. and Tisdale, M.J. (1993). Requirements for (n-6) polyunsaturated fatty acids in tumour growth. *Br. J. Cancer* **67,** (Suppl. XX) 49.

BACR/ ACP/ BOA Annual Meeting, Sheffield 28th-31st March 1993.

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KINETICS OF THE INHIBITION OF TUMOUR GROWTH IN MICE BY EICOSAPENTAENOIC ACID-REVERSAL BY LINOLEIC ACID

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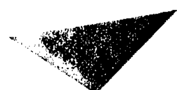

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