TUMOUR ASSOCIATED PROTEOLYSIS AND PROTEIN METABOLISM

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Tumour associated proteolysis and protein metabolism.
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SUMMARY

The effect of cancer cachexia on protein metabolism has been studied in mice transplanted with the MAC16 adenocarcinoma. The progressive cachexia induced by the MAC16 tumour was characterised by a reduction in carcass nitrogen between 16-30% weight loss and a reciprocal increase in tumour nitrogen content. Carcass nitrogen loss was accompanied by a concomitant decrease in gastrocnemius muscle weight and nitrogen content and also by a decrease in liver nitrogen content.

The loss of gastrocnemius muscle througout the progression of cachexia was attributable to a 60% decrease in the rate of protein synthesis and a 240% increase in the rate of protein degradation.

The loss of skeletal muscle protein that may be partially mediated by an increased rate of protein degradation has been correlated with a circulatory catabolic factor present only in cachectic tumour-bearing animals, that degrades host muscle in vitro. The proteolysis-inducing factor was found to be heat stable, not a proteolytic enzyme such as guanidinobenzoatase and was inhibited by indomethacin and eicosapentaenoic acid (EPA) in a dose-related manner. The proteolytic factor induced prostaglandin E_2 formation in the gastrocnemius muscle of non tumour-bearing animals and this effect was inhibited by indomethacin and EPA.

In vivo studies show EPA (2.0g/kg by gavage) to effectively reverse the decrease in body weight in animals bearing the MAC16 tumour with a concomitant reduction in tumour growth. Muscle from animals treated with EPA showed a decrease (60%) in protein degradation without an effect on protein synthesis.

In vivo studies show branched chain amino acid treatment to be ineffective in moderating the cachectic effect of the MAC16 tumour.

The action of the factor was largely mimicked by triarachidonin and trilinolein. The increased serum levels of arachidonic acid in cachectic tumour-bearing animals may thus be responsible for increased protein degradation through prostanoid metabolism.

The understanding of protein metabolism and catabolic factors in the cachectic animal may provide future avenues for the reversal of cachexia and the treatment of cancer.

Key word : Cancer cachexia, MAC16 adenocarcinoma, weight loss, proteolytic factor, protein metabolism.

TO NANNY VEAZEY

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ABBREVIATIONS.

AA	Arachidonic acid.
9AA	9-aminoacridine.
BCAA	Branched chain amino acid.
BSA	Bovine serum albumin.
BZAR	Bis(carbobenzyloxycarbonyl-L-arginamido)-rhodamine.
cAMP	cyclic adenosine 5':3'monophosphate.
$Conc^n$	Concentration.
DHA	Docosahexaenoic acid.
DNS-GGACK	Dansyl-L-glutamyl-glycyl-L-arginine chloro methyl ketone.
DTNB	2-Nitrobenzoic acid.
EDTA	Ethylenediaminetetraacetic acid.
EPA	Eicosapentaenoic acid.
FDE	Fluorescein Di Ester.
FFA	Free fatty acids.
GB	Guanidinobenzoatase.
h	Hour.
IL-1	Interleukin-1.
IL-6	Interleukin-6.
IFN	Interferon.
L	Linoleic acid.
LDH	Lactate dehydrogenase.

LN	Linolenic acid.
min	Minute.
MU	Methylumbelliferone.
MW	Molecular weight.
MUGB	Methylumbelliferyl-p-guanidinobenzoate.
NAD	Nicotinamide adenine dinucleotide.
NADH	Nicotinamide adenine dinucleotide reduced form.
NTB	Non tumour-bearing.
OL	Oleic acid.
P	Probability.
PGE_2	Prostaglandin E_2.
Pi NaCl	Phosphate saline.
PMSF	Phenymethylsulphonyl fluoride.
PUFA	Polyunsaturated fatty acid.
SEM	Standard error of the mean.
Sp.act.	Specific activity.
STI	Soya bean trypsin inhibitor.
tPA	Tissue-type plasminogen activator.
Tris	Tris(hydroxymethyl)methylamine.
TNF	Tumour necrosis factor.
u-PA	Urokinase-like plasminogen activator.
Z-lys-SBzl	Thiobenzyl benzyloxycarbonyl-L-lysinate.

CHAPTER 1: INTRODUCTION.

1.0. Cancer and weight loss.

Cachexia is a debilitating syndrome characterised by progressive emaciation. The term cachexia is a derivation of a Greecian phrase "kakos hexis" literally meaning bad condition (Shamberger, 1984). In many cancers which are difficult to detect weight loss is often the most frequent presenting symptom (Chute et al, 1985) and this weight loss is of considerable prognostic value. The greater the weight loss, the poorer the prognosis and the lower the response rate to chemotherapy (DeWys, 1986). The cachectic cancer patient becomes aware that he has lost control of his weight and may never regain his former physical stature and feeling of well being associated with good nutritional status. The "physical fading of wholeness" is recognised by the individual and is perceived to signal impending death (Costa, 1963).

The occurence of cachexia varies with tumour type and stage of disease ranging from 80-87% in patients with carcinomas of the pancreas and stomach to 48-61% of patients with colon, prostatic and lung cancer. Oesophageal cancer has very high incidence of weight loss. The high incidence of cachexia in cancers of the gastrointestinal tract and related structures is largely due to mechanical interference. It has been recognised that approximately two thirds of cancer patients experience cachexia and at death cachexia was

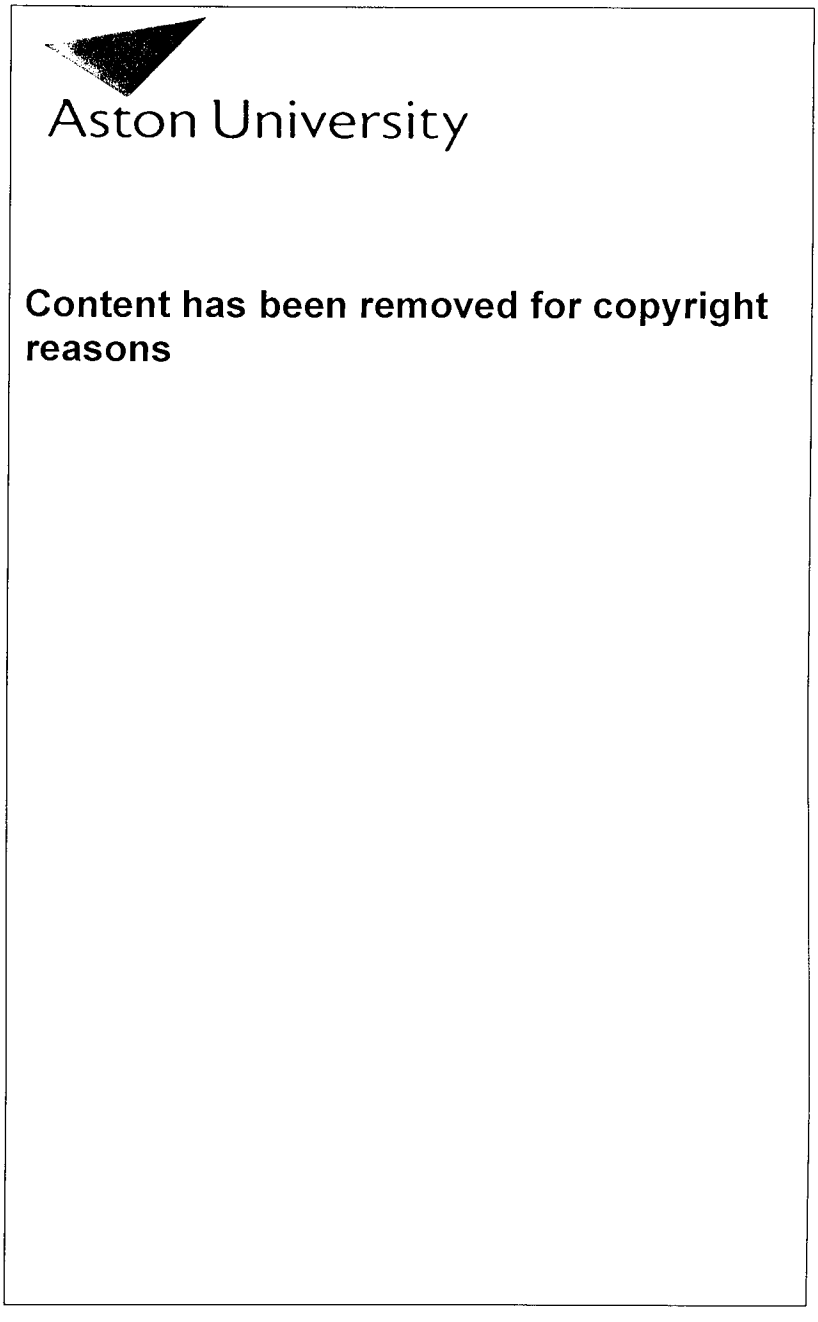
found to be the predominant cause in 66% of cases (Harnet,1932). In 1986 Lindsey ascribed the difference between cachectic and non-cachectic cancer patients to the impairment of some as yet unidentified gene repression however, this theory has not been substantiated.

1.1. The syndrome of anorexia and cachexia.

The causes of weight loss in the cancer patient are multidimensional - these are illustrated in Fig.1. Warren in 1932 described cachectic cancer patients as appearing to die from starvation. Since then numerous metabolic studies have shown this not to be the case (Brennan, 1977) however there is an important link related to starvation - anorexia. The syndrome of anorexia and cachexia is a vicious self-perpetuating cycle that commonly leads to a malnourished, emaciated cancer patient. The two components of the syndrome are not mutally inclusive - it is possible for cachexia to occur in the absence of anorexia (Brennan, 1977 and Lawson et al, 1982). Although anorexia is a common feature in cancer patients (Willcox et al, 1984, found that 43% of cancer patients in one study complained of anorexia), it is not the major consideration in cachexia as force-feeding and hyperalimentation only transiently reversed weight loss (Terepka and Waterhouse, 1956).

1.2. Anorexia.

In a study by DeWys (1985) the Harris Benedict equation was Figure 1.



Factors contributing to weight loss in cancer cachexia.
(Lindsey, 1986)

used to calculate the basal energy requirement for cancer

patients with a moderate level of activity. Of the patients studied only 30% had a caloric intake sufficient to meet the needs for basal energy expenditure and moderate activity whereas 25% of patients had a caloric intake that was insufficient to even meet basal energy demands. The loss of appetite that leads to a decrease in food intake is termed anorexia or hypophagia.

The control of appetite is a complex process integrated in the ventral hypothalamus. Stimulation of the medial hypothalamic nucleus inhibits feeding, while stimulation of the lateral nucleus promotes food intake. A variety of receptors feed sensory input to the appetite control centre - oral stimulation by a pleasant taste would elicit feeding whereas gastrointestinal distention would prevent it. Visceral receptors in the stomach and intestine monitor the temperature, volumetric, osmotic and chemical properties of the food whilst blood levels of glucose, lipids and amino acids are simultaneously monitored to ensure an appropriate response (Shamberger 1984, Balducci and Hardy, 1985).

The pathogenesis of anorexia associated with cancer is complex and cannot be attributed to any single factor. A number of factors contribute to the taste aversion experienced by many cancer patients. DeWys (1978) has reported an elevation of the taste recognition threshold for salt and sweetness, a decreased threshold for bitter

and a low threshold for urea in 15 out of 20 cancer patients and this phenomenon could be correlated with the extent of the tumour. Shamberger (1984) suggests that a zinc deficiency might be the underlying cause in the taste alterations seen in cancer patients and these may be related to the food aversions and cravings experienced during pregnancy.

Another problem that is commonly encountered by the cancer patient is early satiety - this may be due to two major factors. Knox (1983) proposed that a sustained stimulation of the gastrointestinal tract would result as a consequence of tumour-bearing and this would lead to prolonged satiety and a decreased stimulation of appetite. In addition a high proportion of cancer patients have a diabetic glucose tolerance curve as a result of abnormal, i.e. depressed or delayed insulin production, insulin resistance or decreased insulin receptor affinity (Kisner et al, 1978). Consequently blood glucose levels remain abnormally high for a prolonged length of time supressing appetite. The decreased serum insulin levels are also thought to inhibit appetite (Grossman et al, 1947).

Increased blood lactate levels may be an anorexigenic factor (Baille et al, 1970). De Wys (1985) has shown that the infusion of lactate into normal volunteers produces symptoms of anorexia, nausea and anxiety. Increased

blood fatty acid levels and alterations in the amino acid profiles of cancer patients may also contribute to anorexia (Waterhouse et al, 1964 and Krause et al, 1979). Krause et al (1979) found an increase in brain tyrosine in rats bearing the Walker 256 carcinoma - tyrosine is a catecholamine precursor and this may suggest the involvement of catechoalamine neurotransmitters in the development of anorexia.

The anorexia associated with the cancer patient may further be exacerbated by mechanical interference with the ingestion, digestion, absorption and assimilation of food caused by malignancies of the head, neck, oesophagus and gastrointestinal tract. This represents not only a local effect but also a systemic effect caused by the presence of a tumour at a distant site (Schein et al, 1975 and Kelley et al, 1961).

Unfortunately the side effects commonly associated with anticancer therapies - fear, nausea, vomiting, diarrhoea, sore throat and dry mouth together with the pain caused by the disease may contribute substantially to anorexia (Donaldson et al, 1979).

An all encompassing hypothesis was proposed by Theogolides (1972) to account for anorexia - this involved the tumour secreting peptides, oligonucleotides and other small

metabolites that had a peripheral and direct effect on the hypothalamic control of feeding. However, the destruction of the ventromedial hypothalamus - the satiety control centre does not prevent cachexia in tumour-bearing animals (Liebelt et al, 1971). Tumour necrosis factor α (TNFα) or cachectin, produced by macrophages was originally implicated as being the mediator of wasting in chronic diseases (Cerami et al, 1985). However, it is now thought to play a major role in the pathophysiology of anorexia rather than cachexia (Mahony and Tisdale, 1988, Stovroff et al, 1988) as there has been no correlation between wasting and TNFα levels.

1.3. The metabolic implications of anorexia and starvation.

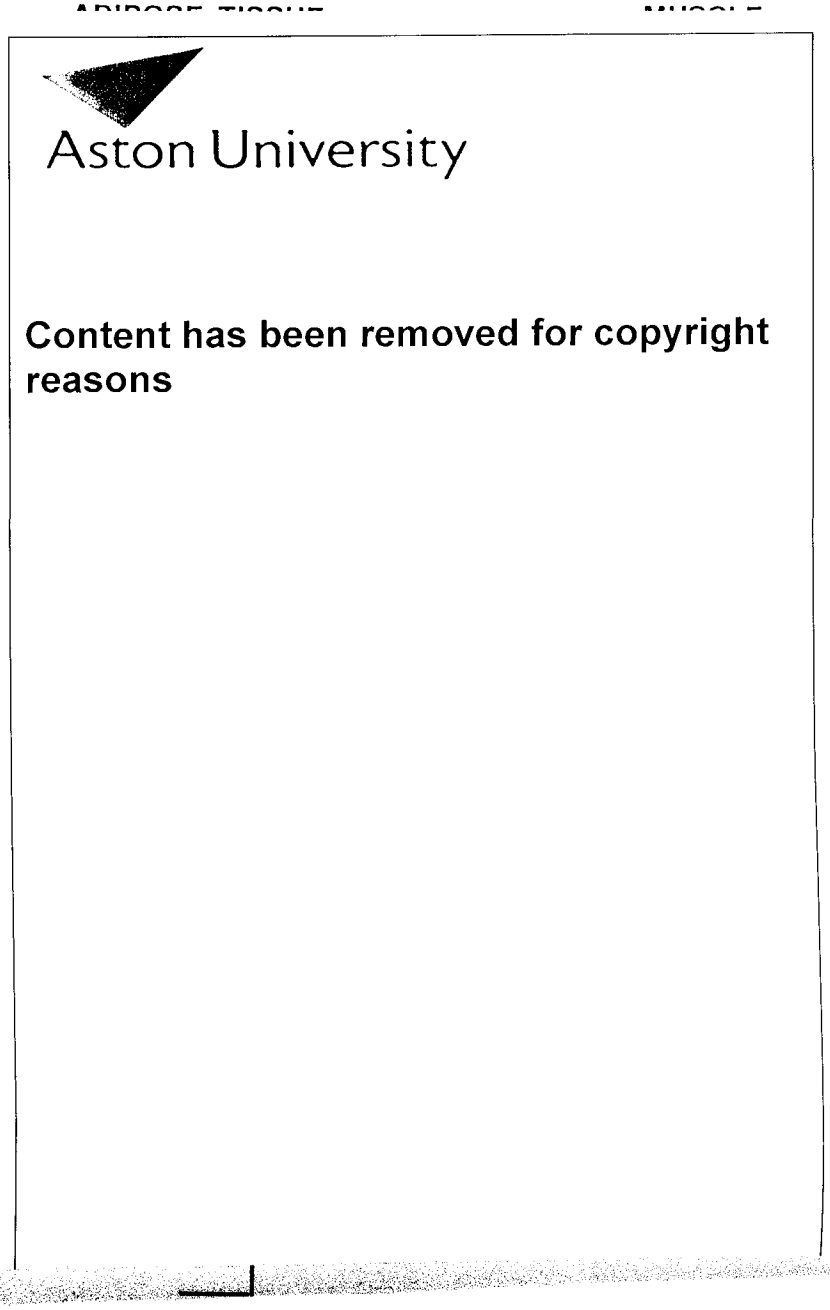
In man the prolonged deprivation of food leads to adaptive metabolic changes that prevent rapid death. Survival is dependent upon the conservation and frugal metabolism of fuel reserves. Fat is the major fuel depot in man and is consequently the primary source of energy during starvation. In a study by White et al (1984) obese volunteers were starved for in excess of six months - and survived. During the initial or acute phase of anorexia or starvation there is a decrease in blood glucose levels with a concomitant decrease in plasma insulin and a rise in plasma glucagon concentration. Glucose levels are

maintained initially to satisfy the glucose dependency of the brain by rapid proteolysis with amino acid mobilization (alanine), increased gluconeogenesis in the liver and then the kidney and an accompanying increase in the output of urinary urea nitrogen (Tisdale, 1982). If this situation continued unabated 30% muscle mass would be lost in 20 days leading to death (Brennan, 1977). Consequently the body adapts to using alternative fuels (Fig.2). The rise in the glucagon/insulin ratio mobilises free fatty acids (FFA) from adipose stores to be used as a source of energy by peripheral tissues. Excess FFA are oxidised in the liver to form ketone bodies (acetoacetate and 3-hydroxybutyrate) (Fig.3). During prolonged starvation the brain together with other tissues can adapt to metabolise ketone bodies thus sparing limited glucose supplies and lean body tissue through a decrease in gluconeogenesis. After a month of starvation 50-60% of the brains' energy requirement is supplied by ketone bodies. Ketone bodies are conserved by renal tubular reabsorption to maintain high circulating levels. Lipid stores are conserved as a direct consequence of ketone bodies reducing lipolysis in adipose tissue (Bjorntorp, 1966) and also by stimulating insulin production. In addition, in prolonged starvation, ketone bodies decrease plasma levels of urinary nitrogen excretion (Sherwin et al, 1975) and alanine through an inhibition of branched chain amino acid oxidation (Buse et al, 1972). The role of ketone bodies in

prolonged starvation is shown in Fig.4.

Survival of the starving patient is dependent on minimizing protein catabolism - this is done through an adaptive

Figure 2.



Adaptations to the starving state.

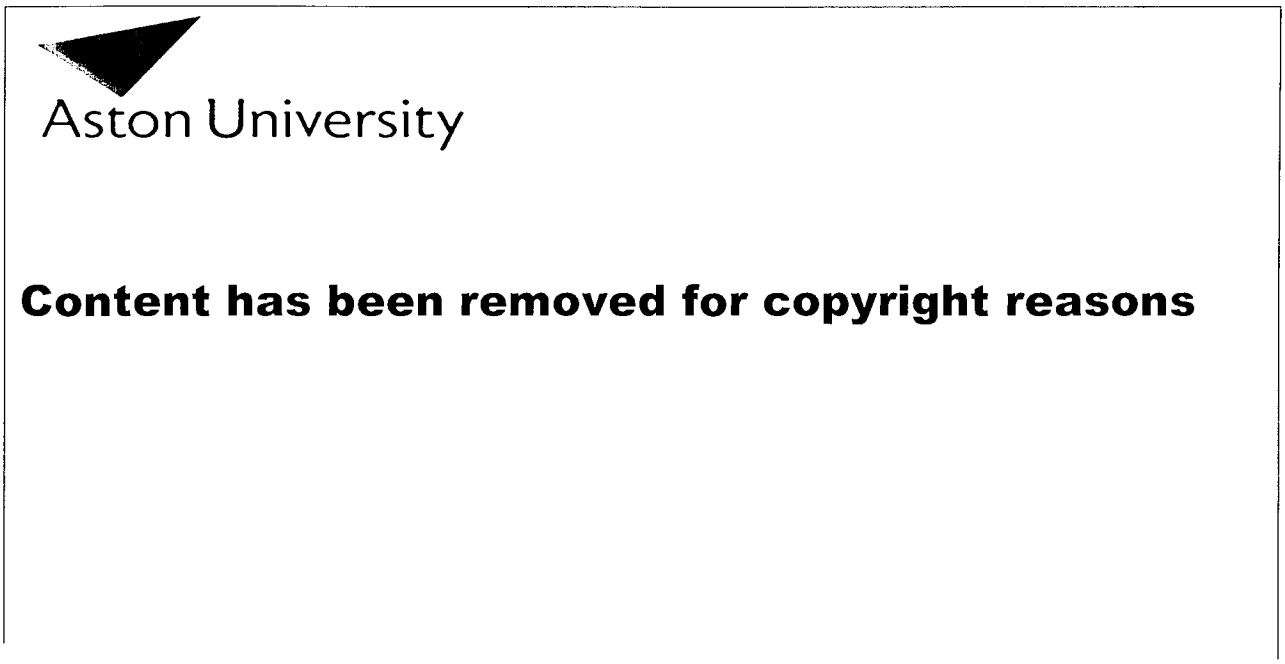
(Tisdale, 1986)

Figure 3.

The formation of ketone bodies from acetyl CoA.

(Bowman and Rand, 1980)

Figure 4.



The role of ketone bodies in prolonged starvation.

(White et al, 1984)

mechanism. Glucose is replaced by ketone bodies as the most important oxidative fuel utilized by the brain, and there is a concomitant decrease in hepatic gluconeogenesis. The resultant fall in oxidative metabolism, metabolic rate, as well as carbon dioxide output leads to a lowering of the respiratory quotient. This "fat fuel economy" will permit a person to survive for long periods with little or no food (Shamberger, 1984).

1.4. Cachexia.

Cachexia is a complex metabolic condition that results in profound debility and malnutrition - the degree of which often seems to far exceed that which might be expected on the grounds of the extent of the neoplastic process (Tisdale, 1986). At autopsy the tumour usually accounts for less than 5% of total body weight (Lindsey,1986). Cachexia is not directly correlated with food intake or the location, the size or the histological type of the tumour. In 1963 Costa defined cachexia as "the sum of those effects produced by neoplasms in the host, which are not the immediate result of mechanical interference with recognizable structures". Cachexia is characterised by the following symptoms;

1. Anorexia and nausea.
2. Weight loss.

3. Anaemia.
4. Altered host metabolism.
5. Muscle weakness.

In addition many cancer patients are susceptible to infection due to impaired immune function; they also experience decreased motor and mental skill and a decline in their attention span.

A number of differing theories have evolved to suggest the cause of cancer cachexia and these can be divided into four groups;

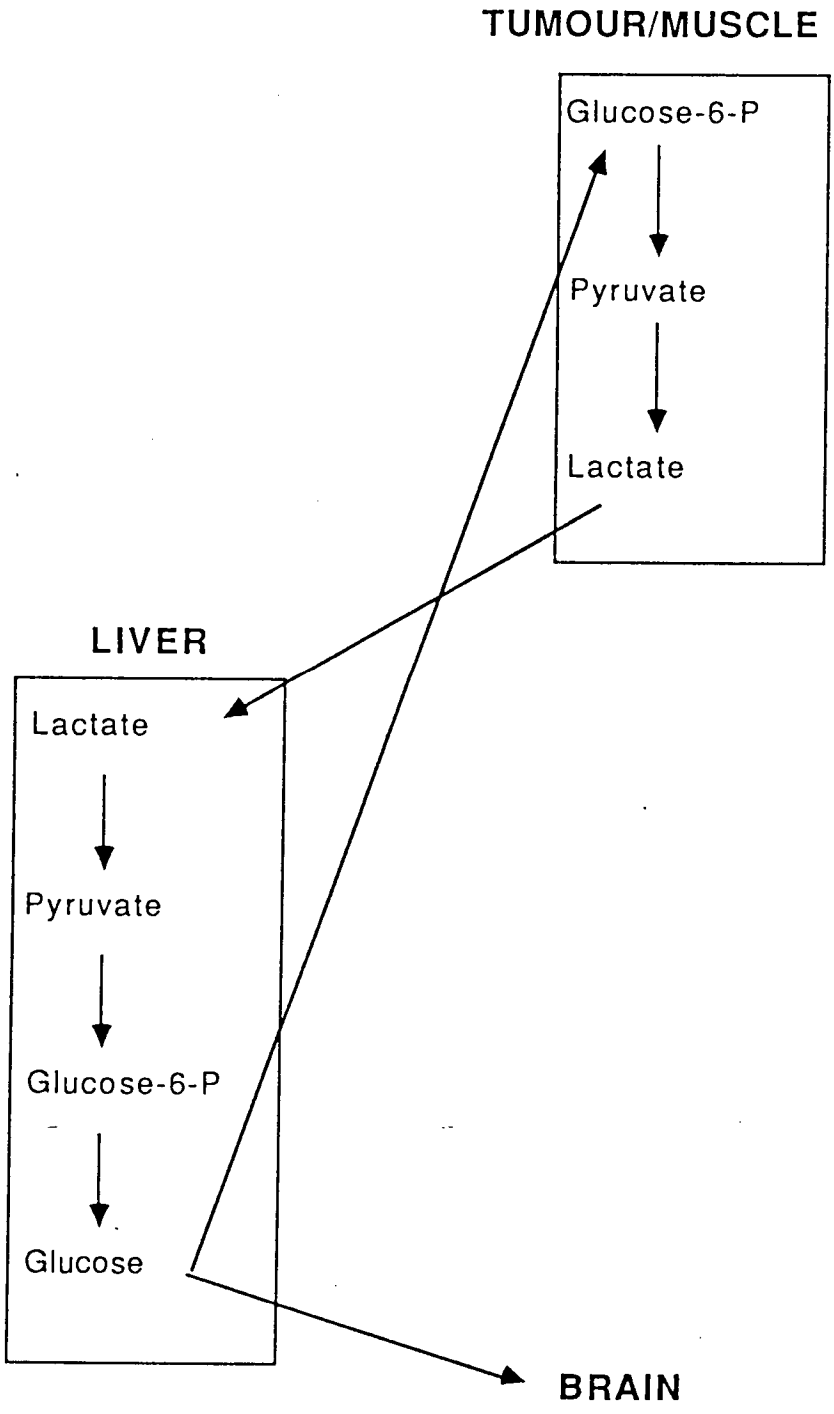
1. Anorexia and defects in absorption.
2. Increased energy expenditure and alterations in host metabolism.
3. Circulating tumour by-products.
4. Tumour metabolism.

In cancer cachexia the adaptive mechanisms that are present in the starved state that lead to the conservation of body stores and a lowered metabolic rate are not in operation. Indeed some cancer patients exhibit a hypermetabolic rate - this puts an additional strain on the cachectic patient who not only has at least to maintain food intake but also has to considerably enhance it in an attempt to maintain weight or to moderate loss. It has been suggested that cachexia may be due to a failure of food intake to compensate for

the increased metabolic rate - however this has largely been refuted by the results of hyperalimentation studies which suggest that cancer patients do not respond as well as malnourished patients to nutritional supplementation (Nixon et al, 1981). In addition if weight gain is achieved it is usually due to fat deposition and extracellular fluid retention (Cohn et al, 1982, Terepka and Waterhouse, 1956) rather than through the replenishment of lean body mass. Tumour growth may also be selectively accelerated by hyperalimentation (Balducci and Hardy, 1985).

In 1968 Gold proposed that cachexia was the result of excessive host gluconeogenesis in an attempt to maintain glucose homeostasis in the face of the tumour inefficiently metabolising glucose via the Cori cycle (Fig.5). The tumour derives little energy from the glucose metabolism whereas the host expends considerable energy in its resynthesis. The Cori cycle produces two ATP molecules whilst gluconeogenesis requires six ATP molecules. As the tumour grows it consumes increasing amounts of glucose, and the energy reserves of the host are depleted in a futile effort to maintain blood glucose levels within the normal range. However this theory does not explain how small tumours can produce cachexia and why some tumours with low Cori cycle activity do produce cachexia in the cancer patient (Ainsenberg, 1961).

Figure 5.



The Cori cycle.

In 1972 Theogolides attributed the cause of cachexia to the production of metabolites by the tumour that allosterically activated or inactivated various enzyme systems in the host - resulting in metabolic chaos. However to date no substances with these properties have been identified.

Cachexia has also been explained in terms of host depletion as a result of tumour parasitism - the tumour being metabolically more aggressive, preferentially sequestering host nutrients (Stein, 1978). However this hypothesis does not explain how some cancer patients with tumour burdens of less than 0.01% of their body weight develop cachexia (Lindsey, 1986).

Williams and Matthaei (1981) proposed that a whole sequence of events that accompanied tumour-bearing were responsible for the initiation and maintenance of the cachectic state. These events are:

(i) Tumour induced retrodifferentiating change in the liver resulting in massive decreases (97%) in the activity of alanine aminotransferase in the organ.
(ii) High concentrations of alanine in the blood which arise from muscle and whose blood concentration is stoicheiometrically-dependent on the oxidation of branched-chain amino acids.

(iii) Increased liver gluconeogenesis from lactate, glycerol but not from the carbon chains of gluconeogenic amino acids.

(iv) Specific metabolic stress resulting from the above abnormal metabolism and characterised by increased secretion of vasopressin (antidiuretic hormone).

(v) Suppression by vasopressin (10nM) of long chain fatty acid oxidation and of ketone body production (65%) decreased.

(vi) Insufficient blood concentration of ketone bodies to regulate branched chain amino acid oxidation by muscle.

This course of events is proposed to lead to the situation where there is chronic muscle breakdown characterised by alanine release, the liver is unable to "rescue" the alanine for gluconeogenesis as a result of lack of alanine aminotransferase in the liver - this leads to irreversible loss of muscle nitrogen in the urine. In addition the inhibition of ketone body production as a consequence of stress related vasopressin release exacerbates branched chain amino acid oxidation and alanine release from muscle. Williams and Matthaei (1981) postulated that ketone body infusion or glucagon treatment would reverse the series of proposed events associated with the pathogenesis of cachexia. Ketone body infusion may be a partial solution to the problem (see section 1.8.). Glucagon treatment would increase glycogen breakdown, inhibit glycogen

synthesis and glucose oxidation and stimulate lipolysis, gluconeogenesis and ketogenesis. The effectiveness of this treatment has not been reported.

There is evidence that both the anorectic and cachectic response of the host are the result of circulating factors/ intermediary metabolites produced by the tumour. This evidence came largely from parabiotic experiments where the circulation of a non tumour-bearing rat was linked to that of a tumour-bearing cachectic rat (Norton et al, 1985). The result was that cachexia and anorexia were conferred upon the non tumour-bearing parabiotic half and this effect was not a consequence of metastasis or endocrine function.

The hosts immune response to the tumour has recently been under scrutiny - in particular a variety of cytokines responsible for the amplification of the secondary systemic reaction of the acute phase response i.e. interleukin-1, interleukin-6, interferon and tumour necrosis factor α (section 4.6.1.).

It is probable that some elements from these theories are involved in the pathogenesis of cachexia - however as yet no single plausible theory can account for all observed cases of cachexia.

1.5. Cachexia associated with trauma.

Trauma can be classified as any condition that causes distress - consequently a number of disease states together with injury may be associated with cachexia. Weight loss can be as great as 30% after hospitalisation for multiple fractures or as little as 6% after uncomplicated surgery (White et al, 1984). Patients are often subjected to a combination of injury and infection thus exacerbating the situation. Indeed sepsis following surgery is one of the leading causes of death in the post-operative patient. In addition diseases such as alcoholism, tuberculosis, emphysema, diabetes and cardiovascular disease may also elicit weight loss to some degree and this is often associated with muscle wasting. Apart from metabolic disorders such as diabetes a basic mechanism is thought to exist to account for trauma-related cachexia. Initially there is a shock/stress phase that stimulates adrenaline release as part of the flight or fight response. The injury may itself stimulate the hindbrain and hypothalamus to release a number of hormone releasing factors. These act on the pituitary to release hormones such as adrenocorticotrophic hormone (ACTH), prolactin, thyrotrophin and growth hormone. These hormones acting directly or indirectly amplify the normal endocrine response and this leads to a general mobilisation of available metabolic substrates leading to weight loss. This leads to the observation of hypermetabolism, negative

nitrogen balance and hyperglycemia in the traumatised patient. In addition this hypermetabolic phase is characterised by increased respiration and heat production. These effects are further accentuated in the burns patient. Weight loss in these patients is considerable due to evaporative water loss and radiative heat loss together with an energy requirement that may exceed 5000 Kcal per day (Curreri and Luterman, 1978). Thus general weight loss in the traumatised patient is due to a complex interaction of hormonal factors, metabolic rate and a failure of the exogenous calorie intake to compensate for the rapid consumption of endogenous fuel reserves - however the role of the catabolic hormones in protein metabolism has not been fully established.

Severe trauma and sepsis are characterised by accelerated degradation and reduced synthesis of skeletal muscle protein and in addition there is inhibition of muscle amino acid uptake (Hasselgren et al, 1984). Warner et al (1990) infused a mixture of catabolic hormones comparable to those found in the traumatised and septic patient into rats. This resulted in elevated glucose and lactate levels, reduced concentrations of most amino acids and accelerated muscle protein breakdown - however protein synthetic rates and amino acid uptake in incubated muscles were not significantly different from the controls. Thus it was concluded that catabolic hormones partially mediate protein

metabolism by enhancing muscle proteolysis but the reduced protein synthesis and amino acid uptake were probably signalled by another mechanism. Reduced protein synthesis may be the result of bed rest - in 1954 Shonheyder et al completely immobilised volunteers in plaster casts. This resulted in a negative nitrogen balance of 10g/d and a 15-20% decrease in protein synthesis. The increase in protein degradation in trauma and sepsis has been attributed to a number of additional mechanisms including the production of a proteolysis inducing factor (Clowes et al, 1983), the action of prostaglandins (section 4.6.1.) and to changes in proteinase/proteinase inhibitor levels in skeletal muscle (Kuehn et al, 1988).

1.6. The metabolic implications of cachexia.

Cachexia is an extremely complex metabolic disorder and in order to assess the impact of tumour-bearing on host metabolism it is necessary to view tumour and host as distinct metabolic entities (Costa, 1963).

1.6.1. Tumour metabolism.

If the host has a slow growing, non-metastasizing, discrete tumour it is tempting to view it as a rather inert protoplasmic addition living as a commensal organism sharing host resources. However this is not the case, the

tumour exists in a niche more akin to the parasite, acting as a metabolic trap sequestering nutrients to the detriment of the host. The tumour is able to preferentially concentrate amino acids from the hosts plasma pool (Wiseman and Ghadially, 1955, Lazo, 1981) and in this way the tumour acts as a nitrogen trap competing with the host for nitrogen compounds (Mider, 1951). It has been shown that some tumours show a marked dependence on or a preference to a particular amino acid - for example the Lewis lung carcinoma favours glutamine utilisation (Rivera et al, 1988) and this phenomena may lead to the development of treatment regimes where the tumour is selectively starved. However, this selectivity can lead to a negative nitrogen balance in the host as a result of reciprocal tumour nitrogen gain or as a consequence of non-essential amino acid waste (Tisdale, 1986).

Tumours are avid users of glucose as a metabolic substrate. In many situations, however, the tumour is poorly vascularised and oxygen diffusion is limited to 150μm from a blood vessel. Consequently large solid tumours contain many hypoxic cells and under these conditions glucose is metabolised by the Embden-Meyerhoff pathway to lactate. In 1930 Warburg demonstrated that many tumours metabolise glucose via glycolysis at a high rate even in the presence of normal oxygen availability. The increased tumour glycolytic activity results in an increase in lactic acid

production leading to host lactic acidosis (Shamberger, 1984). Jain et al (1984) demonstrated that the outpouring of lactate caused a pH decrease from 7.3 to 6.2 in the rat Walker 256 carcinoma with increasing tumour mass up to 50g. The reason that the anaerobic pathway of glucose metabolism is favoured may be due to the high activity of lactate dehydrogenase or the low oxygen tension (Lazo and Sols, 1980). This gives a high ratio of lactate dehydrogenase to pyruvate dehydrogenase which encourages pyruvate to take the anaerobic pathway.

Tumour cells in addition have been shown to be able to metabolise lipids (Thomson,1981, Baker et al, 1977). The fatty acids reach the tumour complexed with serum albumin, or in the form of lipoproteins and their entry into the tumour cell is thought to be governed by the activity of lipoprotein lipase. Thomson (1981) found that lipoprotein lipase levels were elevated after starvation in mice bearing a non-metastatic tumour compared to the controls. In animals bearing the MAC16 colon adenocarcinoma fat oxidation as measured with [U-^{14}C]palmitic acid and [1-^{14}C]triolein is significantly increased when compared to non tumour-bearing controls suggesting that the mobilisation of host lipid stores is accompanied by an increase in lipid utilisation (Mulligan and Tisdale, 1991). The relative importance of fatty acids, glucose or amino acids as substrates for tumour metabolism varies according

to the state of development and the type of tumour (Pederson, 1978).

1.6.2. Host metabolism.

Cachectic cancer patients often possess a hypermetabolic rate - this is in stark contrast to the starved state where hypometabolism predominates. However, it has not been fully established if the resultant cachexia can be attributed to insufficient food intake failing to meet increased energy demands. What is certain is that the tumour continues to grow as host tissues dissipate giving the appearance that host tissues are in a state of accelerated starvation. This may possibly be as a result of the tumour eliciting amongst other effects an inappropriate response to starvation (Argiles and Azcon-Bieto, 1988). The tumour-bearing state also induces profound changes in host protein, carbohydrate and fat metabolism.

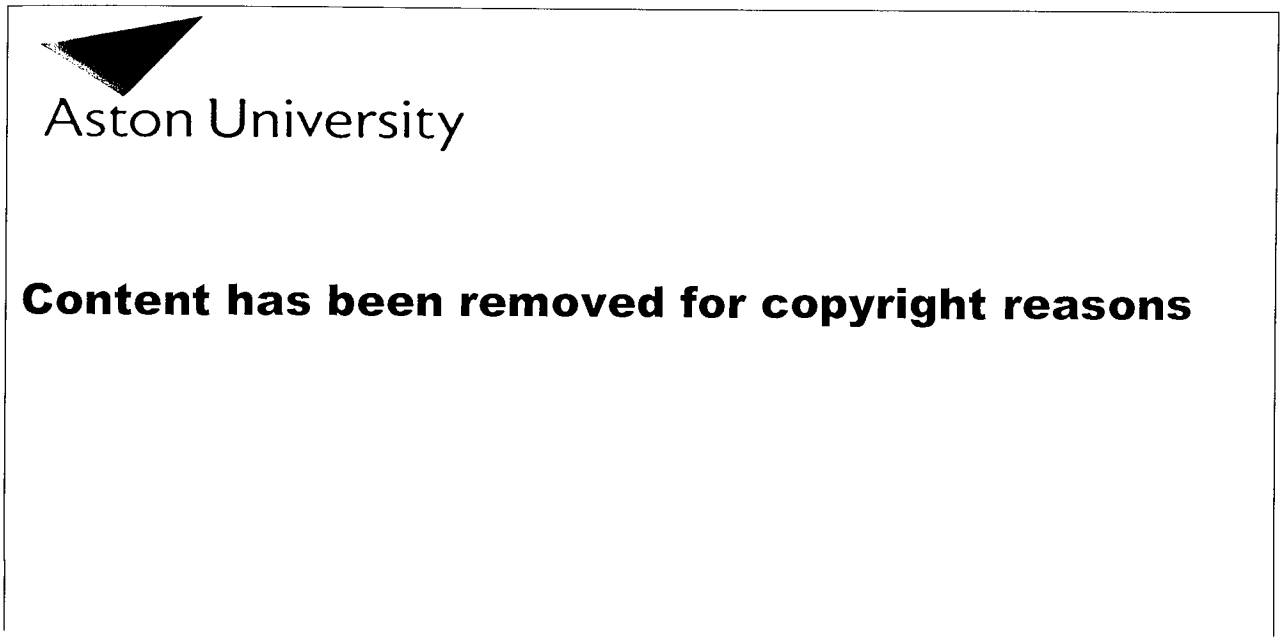
1.6.2.1. Alterations in protein metabolism.

The control of protein turnover, i.e. the continual breakdown and renewal of proteins is complex and is still not well understood. Skeletal striated muscle is composed of elongated muscle cells (Fig.6.) surrounded by an electrically excitable membrane called the sarcolemma.

Each cell contains many parallel myofibrils about 1μm in diameter immersed in sarcoplasm - the intracellular fluid. Each myofibril contains thick (myosin) and thin (actin, tropomysin, and troponin) filaments. These filaments are arranged in repeating "A" and "I" bands. Within the "A" band an "H" zone exists containing only thick filaments, the "I" band is composed of thin filaments and the remainder of the "A" band consists of overlapping thick and thin filaments. The contractile activity of the muscle is largely controlled by active sequestration of Ca^{2+} by the sarcoplasmic reticulum - depolarisation of the transverse tubule membrane causes a sudden release of Ca^{2+} from the sarcoplasmic reticulum initiating the process of muscle contraction (Stryer, 1981).

Proteins of myofibrillar origin account for about 70% of muscle the remainder are located solubilised in the sarcoplasm. In the steady state, turnover necessitates the removal, degradation, and resynthesis of myofibrillar components whilst maintaining the muscles structural integrity and contractility (Millward, 1980). The rate of protein turnover for specific elements within the muscle varies - for example structural proteins of the "Z"-band (a-actinin) and "M"-line ("M" line proteins) turn over rapidly whereas the thin filament proteins turn over more slowly (Millward, 1980). There appears to be two routes by which intracellular proteins can be catabolised - lysosomal

Figure 6.



A schematic diagram of skeletal muscle.

(Stryer, 1981)

and non-lysosomal. The role of lysosomes in the regulation of protein breakdown is not clear. Lysosomes though not abundant are present in skeletal muscle and their purified proteinases, cathepsins B, D, H and L have been shown to degrade purified actin and myosin to a limited degree (Bird et al, 1980). However Lowell et al (1986) have shown that lysosomal proteolysis is not a prominent feature in myofibrillar protein breakdown but it may contribute to non-myofibrillar proteolysis. It has also been demonstrated that increases in muscle protein degradation seen with arachidonic acid, prostaglandin E_2, high Ca^{2+} levels and in the unrestrained muscle can all be inhibited with leupeptin or Ep475 - inhibitors of lysosomal proteases (Rodemann and Goldberg, 1982, Goldberg et al, 1984, Baracos et al, 1986, Baracos and Goldberg, 1986).

Another component of the muscle degradation system is the calcium-activated neutral proteinase, which as its name suggests requires calcium for substrate binding and is active at neutral pH. Goll et al (1978) demonstrated that this proteinase can rapidly remove the "Z"-line and disrupt the "M"-line in the myofibril - these structures are ostensibly involved in anchoring the thick and thin filaments together and are consequently important in maintaining the three-dimensional structural integrity of the myofibril. The precise location of this proteinase within the muscle has not yet been established. However,

it is thought that myofibrillar protein turnover may be initiated by this calcium-activated neutral proteinase (Millward, 1980).

Alkaline proteinases have been implicated in myofibrillar protein degradation - Dahlmann and Reinauer (1981) have shown that in the streptozotocin-diabetic rat muscle alkaline proteolytic activity increased in parallel with protein degradation. It is now known that the majority of activity associated with alkaline proteinases can be attributed to chymase which originates from intramuscular mast cells (Dahlmann et al, 1982).

In addition in muscle there is a trypsin-like serine proteinase that was originally isolated from smooth muscle (Beynon and Kay, 1978) that inactivates enzymes in their native conformation and readily digests most myofibrillar proteins (Kay, 1980, Kay et al, 1982). Finally there are group-specific or serine proteinases that also inhibit pyridoxal phosphate containing enzymes and may participate in myofibrillar protein degradation (Millward, 1980). Although these systems have been identified their precise role in protein degradation in even the steady state have not been fully elucidated.

Protein synthesis is controlled in a number of ways - changes in protein synthesis can be effected by a variation

in the amount and activity of the synthetic apparatus, i.e. number and activity of ribosomes. Henshaw et al (1971) concluded that the decrease in protein synthesis associated with starvation reflected both a loss of ribosomes and a decrease in the activity of the remaining ribosomes. Similarly Ashford and Pain (1986) showed that in the diabetic rat the marked decrease in muscle protein synthesis was associated with a pronounced increase in ribosome degradation leading to loss - however, muscle protein degradation was only marginally elevated. It is interesting to note that Sameshima et al (1981) concluded that both lysosomal and non-lysosomal pathways exist for ribosome degradation and that the relative importance of these can vary with physiological conditions. Further mechanisms of synthetic control exist, for example, there can be regulation of translation at the level of initiation. It has been shown that the removal of an essential amino acid from culture medium results in a rapid decline in protein synthesis concomitant with polyribosome disaggregation. This effect is achieved within an hour of deprivation and is completely reversible when the amino acid is refed (Van Venrooij et al, 1972). In addition there are complex mechanisms involving polypeptide chain initiation and variations in the concentration of, and the efficiency of translation of mRNA species that also regulate protein synthesis (Pain and Clemens, 1980).

Cachexia is characterised by depletion of lean body mass (Nixon et al, 1980). The basis for this loss is proposed to be either depressed protein synthesis (Emery et al, 1984, Rennie et al, 1983), increased protein degradation (Tessitore et al, 1987) or generally increased whole body protein turnover (Kien and Camitta, 1983, Jeevanandam et al, 1984). Goodlad and Clark (1972) have suggested that the protein depletion seen in the gastrocnemius muscle of rats bearing the Walker 256 carcinoma was attributable to a 64% decrease in the synthetic capabilities of the polyribosomes - however, in the soleus muscle where protein depletion was not as evident polyribosome activity was 84% of the control value. In addition inadequate protein synthesis may be exacerbated by inactivity (Shonheyder, 1954). Indeed Daneryd et al (1990) have demonstrated that in tumour-bearing Wistar Furth rats exercise delayed anorexia and although not preventing weight loss through depletion of lipid stores, lean body tissue was preserved via an increased RNA/protein quotient - tumour weight was also decreased compared to the controls. However, this effect can only postpone the inevitability of cachexia.

The increased protein degradation that may be evident in the cachectic cancer patient is probably due to a combination of factors - some elements may be similar to those seen in trauma and starvation (section 1.3., 1.5.). There are also the effects of tumour and host metabolism

(section 1.6.1., 1.6.2.), the stimulation of skeletal muscle catabolic enzymes (section 4.1.1.), the possible effect of a circulating mediator (section 4.1.-4.6.) and the influence of branched chain amino acids (section 4.7.).

1.6.2.2. Alterations in carbohydrate metabolism.

Tumours with a high requirement for glucose impose a metabolic stress on the host to maintain glucose homeostasis. Despite the tendency towards hypoglycaemia, normal blood glucose concentrations have been observed in most tumour-bearing animals and patients (Tisdale, 1986). However, Roh et al (1984) demonstrated that in rats bearing a MCA-induced sarcoma, blood glucose levels decreased with increasing tumour burden - this caused a 27% increase in hepatic gluconeogenesis from alanine and lactate, but this was insufficient to meet tumour glucose demands. Thus in order to attempt to maintain glucose homeostasis gluconeogenesis from non-carbohydrate precusors is enhanced (Shapot and Blinov, 1974). Lundholm et al (1982) has demonstrated that gluconeogenesis from glycerol is increased in cancer patients - however, this only contributes 3% to the glucose turnover rate in both cancer and control patients. It is interesting to note that Clofibrate, a blood triglyceride lowering drug, reduces the growth of the Walker 256 carcinoma in rats possibly by making less glycerol available for conversion to glucose

(Gold, 1978). Similarly Waterhouse et al (1979) showed an increase in gluconeogenesis from alanine in the cachectic cancer patient. Holroyde et al (1975) demonstrated that patients with metastatic carcinoma and progressive weight loss exhibited increased Cori cycle activity whereas patients without weight loss had normal glucose metabolism. Increased Cori cycle activity is associated with a concomitant increase in lactate production. Lactate is converted to glucose via a particularly inefficient process that utilises 6ATP molecules per futile Cori lactate-glucose cycle in the liver. Although increased gluconeogenesis in itself is not responsible for high energy expenditure in cancer patients it is thought that an increased rate of glucose turnover may contribute to this effect.

Glucose intolerance in cancer cachexia may be due to abnormal insulin production and insulin resistance, without a reduction in insulin receptors in peripheral tissues (Kisner et al 1978). The use of insulin in the nutritional management of cachectic cancer patients has been suggested - however there are problems with the existing insulin resistance and problems associated with insulin treatment (Schein et al, 1979). Beck and Tisdale (1989) have shown that insulin treatment can minimise weight loss in cachectic MAC16 tumour-bearing mice but unfortunately the treatment also stimulates tumour growth.

1.6.2.3. Alterations in lipid metabolism.

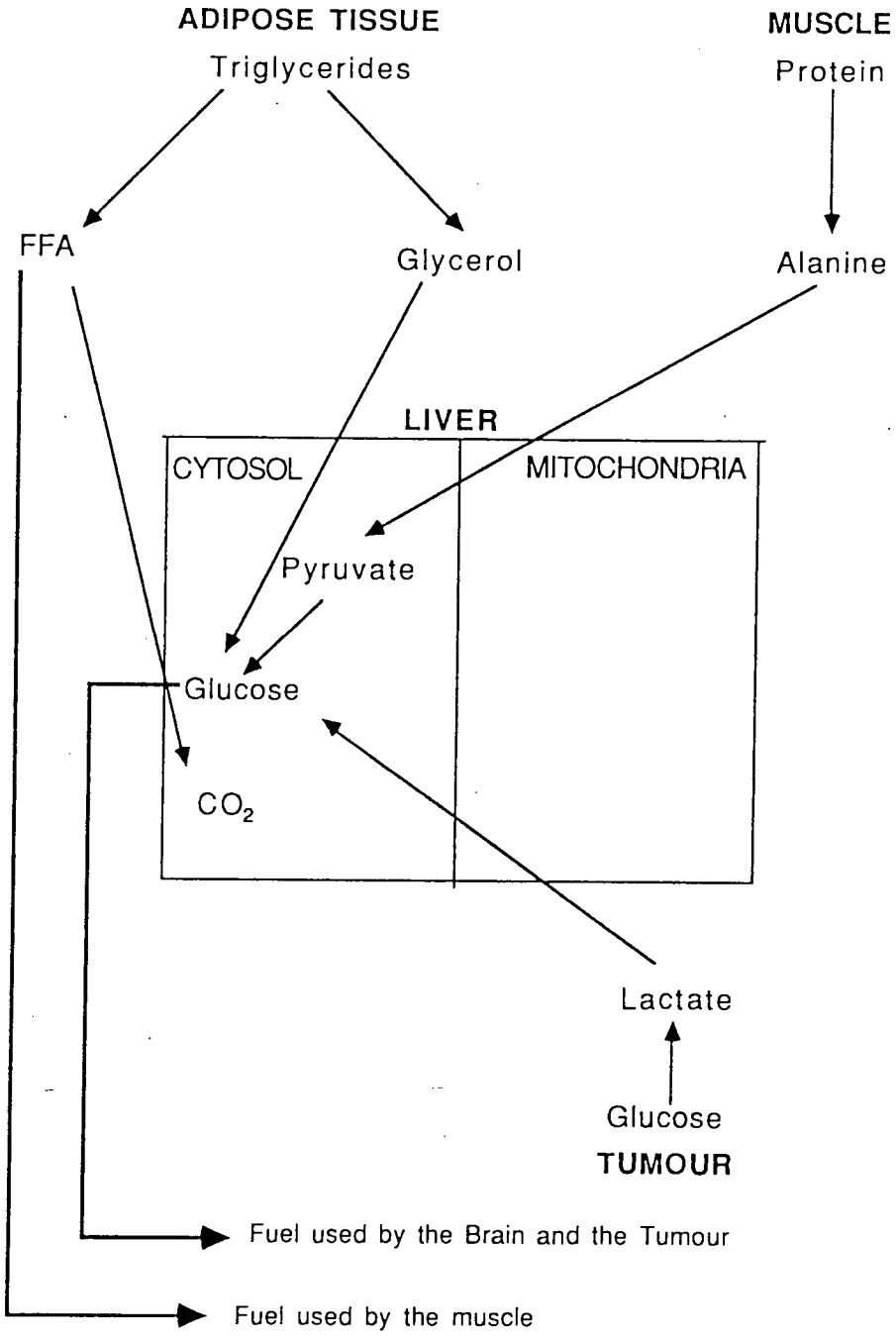
Cachexia is characterised by a progressive decrease in carcass lipids as a result of free fatty acid mobilisation from host adipose tissue. This may be associated with increased plasma lipids as a consequence of a decreased rate of triacylglycerol clearance (Redgrave et al,1984). Indeed, Frederick and Begg (1956) have demonstrated that the weight loss seen in tumour-bearing animals associated with lipid depletion is accompanied by an increase in the plasma level of free fatty acids. However, only 1% of these fatty acids are utilised by the tumour - it is thought that the remainder are oxidised outside the tumour mass probably in the muscles. The degree of hyperlipidaemia may represent the capacity of the extra-tumour tissues to oxidise fatty acids (Tisdale, 1986). The alternative fuel sources used by the cachectic cancer patient are shown in Fig.7.

The mobilisation of free fatty acids is the result of a combination of factors. The problem of insulin resistance may favour increased lipolysis and inhibit lipid deposition, in addition there is the problem of the inability of the cachectic patient, under normal conditions, to develop ketonaemia. Ketosis is a common phenomena in the starving patient and results in the

metabolic adaptations that enable the body to conserve protein and lipid and maintain glucose homeostasis. This allows a starving man to survive for long periods with chronic food deprivation (section 1.3.). However, in cachexia this adaptation does not occur and ketosis is extremely rare. This is further emphasised by the fact that ketonuria in cachectic cancer patients and tumour-bearing rats is similarly uncommon (Conyers et al, 1979, Mider, 1951). It is interesting to note that if a cachectic patient is given an exogenous supply of fatty acids ketonaemia is observed. This suggests that there is no defect in the capability of the cachectic liver to synthesize ketone bodies (Magee et al, 1979). It would appear that many of the features associated with cachexia may be attributable to the absence of ketosis - for example muscle and fat depletion and decreased insulin secretion.

However, this is probably not the entire story as various factors have been identified that may contribute to the lipid mobilising effect. An example of a factor causing lipolysis is Toxohormone-L - this has been isolated from the ascites fluid of mice with sarcoma 180 as well as from the ascites fluid of patients with hepatoma, Grawitz's tumour, ovarian tumours and from the pleural fluid of patients with malignant lymphoma (Masuno et al, 1980). More recently a lipid mobilising factor has been identified that is produced by the experimental murine colon

Figure 7.



Fuel sources in the cachectic cancer patient.

(Adapted from Tisdale, 1986)

adenocarcinoma (MAC16) (Tisdale, 1990). This factor appears to be related as regards charge and molecular weight to a serum factor found in cancer patients experiencing weight loss however the structure remains elusive (Beck, et al 1990). In addition the depletion of host lipid stores may be related to an increase in basal metabolic energy expenditure (Waterhouse, 1981).

1.7. Nutritional support of the cachectic cancer patient.

The proposed reasons for offering nutritional support to the cachectic cancer patient are :-

1. To reverse the cachectic state.
2. To prevent weight loss / maintain weight.
3. To improve improve host tolerance to chemotherapy, radiotherapy or surgery.
4. To improve immune competence.

Unfortunately these criteria are difficult to fulfil. The complexity of the metabolic problems associated with cachexia prevent a simple solution. In theory if calorific intake exceeds energy expenditure weight gain ensues. However a voluntary increase in food intake is difficult to achieve due to the problems of anorexia (section 1.2.). If an increase in calorific intake occurs through voluntary or involuntary i.e. enteral or parenteral feeding the weight gain that may be achieved is usually due to fat deposition or extracellular fluid retention (Cohn et al, 1982,

Shamberger, 1984). Indeed Nixon et al (1981) suggest that the cachectic state decreases the ability of the host to utilise nutrients to replenish lean body tissue. DeWys (1985) recommends that future nutritional support regimes should be combined with an exercise programme in an attempt to rebuild depleted body muscle.

A further complication that may be encountered during nutritional supplementation is the stimulation of tumour growth (Buzby et al, 1980, Balducci and Hardy, 1985). This is also indicated by reports of increased circulating levels of lactic acid during hyperalimentation (Holroyde and Reichard, 1981). The goal of nutritional support is primarily to improve the cancer patients quality of life through maintenance or replenishment of body weight, particularly when this may facilitate effective therapy. However, the use of aggressive nutritional support in a situation in which effective therapy measures have been exhausted is of limited value and may have adverse effects (DeWys, 1985).

1.8. Potentials for future therapy.

Recently there has been a shift of focus from hyperalimentation that may feed the tumour to regimes that preferentially feed the host whilst starving the tumour. This approach exploits a weakness in tumour biochemistry -

for example a leukaemia of T-cell origin is unable to synthesize the non essential amino acid L-asparaginase. Thus it has been shown that the administration of the enzyme L-asparaginase to deplete the host of L-asparagine may result in selective tumour starvation and remission (Holland and Ohnuma, 1979).

Another weakness that may be exploited is the fact that many solid tumours are unable to utilise ketone bodies as an energy source (Tisdale and Brennan, 1983). Beck and Tisdale (1989) showed that the feeding of a high fat diet to cachectic mice modified nitrogen excretion and that ketone bodies could decrease weight loss and tumour volume in mice bearing the MAC16 adenocarcinoma. Dietary induced ketosis has been shown to increase body weight in cachectic cancer patients, however, it has proved to be ineffective in improving the nitrogen balance (Fearon et al, 1988). Fearon and Carter (1985) have demonstrated that dietary ketosis did not reduce tumour growth rate or prevent weight loss in rats bearing the Walker 256 tumour.

Tumours have recently been shown to produce a variety of catabolic factors (section 4.1.1.) and the characterisation and synthesis of these isolated factors may provide a potential for future therapy. Therapy may involve the inhibition of the factor at the site of production or at the site of action. Alternatively the factor could be

removed from the circulation perhaps by a specific monoclonal antibody. Recently there has been much interest in the clinical efficacy of fish oils and their constituent fatty acids. The polyunsaturated fatty acid (PUFA) eicosapentaenoic acid has been shown to have a pronounced anticachectic/antitumour effect in animals bearing the MAC16 colon adenocarcinoma (Tisdale and Beck, 1991). Currently, attention has been focused on a possible mode of action for this PUFA with the potential for future cachexia therapy.

1.9. Aims of the investigation.

A number of animal models exist that do produce cachexia - however, they are often fast growing rodent tumours that produce weight loss at high tumour burdens. This is not analagous to the human situation where tumour growth is usually much slower and weight loss occurs when tumour burden is relatively small - approximately 1% of total body weight (Costa, 1963). The MAC16 adenocarcinoma is a murine model of cachexia that produces weight loss in recipient animals with a small tumour burden - in addition there is the added benefit that weight loss occurs without the complication of anorexia. Weight loss occurs at a tumour burden of 0.2-0.6g which represents 0.8-2.5% of total body weight - this tumour burden can elicit a 10-30% reduction in host body weight.

The aim of this study was to investigate the effect of cachexia on protein metabolism. The effect of the MAC16 tumour on protein synthesis and degradation was quantified using isolated gastrocnemius muscle and this was related to changes in mouse body composition, particularly regarding the nitrogen component. It had previously been shown (section 4.1.1.,4.6.1.) that some mammalian tumours produce catabolic factors that may be responsible for cachexia. Consequently the possibility was explored that the alterations seen in protein metabolism in the cachectic mouse was attributable to a circulating factor. Initially the tumour associated enzyme guanidinobenzoatase was investigated for its potential role as a circulating mediator in cachexia. Guanidinobenzoatase has been shown to be an arginine selective protease that may participate in the metastatic process (Steven and Griffin, 1988). The potential mechanism of action of the mediator of muscle proteolysis was investigated largely using various drugs and dietary treatments in an attempt to abolish this cachectic effect. A dietary treatment was also evaluated in the cachectic mouse that had previously been shown to moderate protein loss in the trauma patient.

The overall aim of this investigation was to increase the knowledge of the etiology of cachexia in cancer, to increase the understanding of protein metabolism in the

cachectic state and to investigate possible treatments to alleviate cancer cachexia.

CHAPTER 2: MATERIALS.

2.0. ANIMALS.

Pure strain inbred female/male NMRI mice (age 12 - 15 weeks) were bred in the animal house of Aston university, Birmingham. Animals were fed ad libitum a rat and mouse breeding diet purchased from Pilsburys Ltd, Birmingham, UK. and were given free access to water.

2.1. GASES AND AGENTS FOR ANAESTHESIA.

BOC LTD, London, England.

Carbon dioxide

Nitrous oxide

Oxygen

Air:carbon dioxide (95%:5%)

Oxygen:carbon dioxide (95%:5%)

Nitrogen:carbon dioxide (95%:5%)

ICI CHEMICAL INDUSTRIES PLC, Pharmaceuticals division, Macclesfield, Cheshire, England.

Fluothane (halothane)

2.2. CHEMICALS.

AMERSHAM INTERNATATIONAL, Buckinghamshire, England.

L-[4-^{3}H] phenylalanine (sp.act. 28 Ci/mmol).

[5,6,8,11,12,14,15-^{3}H(N)]-Prostaglandin E_2 (sp.act.150 Ci/mmol).

BACHEM LTD, Saffron Walden, Essex, England.

Thiobenzyl-benzyloxycarbonyl-L-lysinate.

BDH CHEMICALS LTD, Poole, Dorset, England.

Ethylenediaminetetraacetic acid, disodium salt.

2-methoxyethanol.

Potassium dihydrogen orthophosphate.

Potassium hydroxide.

Sodium bicarbonate.

Sodium carbonate.

Sodium nitrite.

Trichloroacetic acid.

Triethanolamine hydrochloride

BOOTS THE CHEMIST, Nottingham, England.

Liquid parafin.

FISONS SCIENTIFIC APPARATUS, Loughborough, England.

Chloroform.

1,2-dichloroethane.

Dipotassium hydrogen orthophosphate.

Disodium hydrogen orthophosphate.

Hydrochloric acid.

Magnesium sulphate, hydrated.

Nitric acid.

Perchloric acid.

Sodium dihydrogen orthophosphate.

FSA LABORATORY SUPPLIES, Loughborough, England.

Acetone.

Acetic acid.

Chloroform.

Diethyl ether.

N-N-dimethylformamide.

Ethanol.

Ethyl acetate.

Methanol.

Optiphase Hisafe 3.

Propan-2-ol

Sodium chloride.

Toluene.

GIBCO LTD, Paisley, Scotland.

RPMI 1640 medium, without phenol red.

Dulbecco's minimal essential medium, without phenol red.

SIGMA CHEMICAL COMPANY LTD, Poole, Dorset, England.

L-Alanine.

9-aminoacridine.

Antipain.

Arachidonic acid.

Aspartic acid.

Nα-benzoyl-L-arginine-7-amido-4-methyl-coumarin.

Bovine γ-globulin.

Bovine serum albumin.

Bromcresol green reagent kit.

Calcium chloride.

Charcoal powder, activated (250-300 mesh).

Citric acid.

Copper sulphate.

Cyclohexamide.

Dextran (MW 70 000).

Dimethyl sulphoxide.

5,5'-dithiobis (2-nitrobenzoic acid)

Docosahexaenoic acid

Eicosapentaenoic acid.

Esterase, from rabbit liver.

D-glucose, (dextrose).

Glucose standard solutions

Glycine, sodium salt.

Hydrazine hydrate.

Indomethacin.

Isoleucine.

L-lactate dehydrogenase, from rabbit muscle, type II.

Leucine.

Leupeptin.

Linoleic acid.

Linolenic acid.

Lysine.

4-methylumbelliferone.

4-methylumbelliferyl-p-guanidinobenzoate.

Nicotinamide adenine dinucleotide, grade IV.

Nicotinamide adenine dinucleotide, reduced form, grade III.

O-nitrophenol butyrate.

1-nitroso-2-napthol.

Oleic acid.

Phenylmethylsulfonyl fluoride.

Plasmin, from human plasma.

Plasminogen, from human plasma.

Potassium chloride.

Prostaglandin E_2.

Prostaglandin E_2 antiserum, from rabbit.

Sodium azide.

Sodium glycinate.

Tissue plasminogen activator, single chain from human melanoma cell culture.

O-toluidine reagent.

Triethanolamine.

Tripotassium citrate.

Triton X-100.

Trizma base.

Trypsin, from bovine pancreas, type I.

Trypsin inhibitor, from soybean, type I-S.

Tyrosine.

L-valine.

WEDDEL PHARMACEUTICALS LTD, Wrexham, England.

Multiparin (heparin).

2.3. GIFTS.

The following were kindly donated by :

Dr.F.S.Steven, University of Manchester.

Bis-(N-benzyloxycarbonyl-L-arginamido)-rhodamine.

Dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone.

Tissue plasminogen activator, from human melanoma cell culture.

2.4. BUFFERS.

0.1 M Sodium phosphate buffer (pH 6.0).

Solution A : Sodium dihydrogen orthophosphate (15.60 g) was added to 1000 ml of distilled water.

Solution B : Disodium hydrogen orthophosphate (14.20 g) was added to 1000 ml of distilled water.

Solution B was added to 200 ml of solution A until a pH of 6.0 was reached.

0.01 M Phosphate buffer (pH 8.0).

Solution A : Potassium dihydrogen orthophosphate (1.36 g) was added to 1000 ml of distilled water.

Solution B : Dipotassium hydrogen orthophosphate,

trihydrate (2.28 g) was added to 1000 ml of distilled water.

Solution B was added to 200 ml of solution A until a pH of 8.0 was reached.

0.05 M Tris - HCl buffer (pH 8.0).

Trizma base	3.025 g
Distilled water	400 ml

The pH was adjusted to 8.0 with concentrated hydrochloric acid and the final volume was made up to 500 ml with distilled water.

0.2 M Citrate buffer (pH 5.0).

Citric acid	21.008 g
Sodium hydroxide (1M)	200 ml

The pH was ajusted to 5.0 with 1 M sodium hydroxide and the final volume was made up to 500 ml with distilled water.

Krebs - Ringer bicarbonate buffer (pH 7.6).

0.9 % Sodium chloride	100.0 ml
1.15 % Potassium chloride	4.0 ml
1.22 % Calcium chloride	3.0 ml
2.11 % Potassium dihydrogen phosphate	1.0 ml
3.82 % Magnesium sulphate, hydrated	1.0 ml
1.3 % Sodium bicarbonate	21.0 ml

On the day of the experiment bovine serum albumin 30 g/L and 0.55 mM D-glucose were added to the above.

Krebs - Henseleit bicarbonate buffer (pH 7.6).

This is essentially Krebs - Ringer bicarbonate buffer with the substitution of 1.2 g/L of bovine serum albumin and 6.0 mM D-glucose for the concentrations stated above.

0.4 M Hydrazine - 0.5 M glycine buffer (pH 9.0).

Glycine	11.4 g
Hydrazine hydrate	25 ml
Distilled water	200 ml

The pH was adjusted to 9.0 with concentrated hydrochloric acid and the final volume was made up to 300 ml with distilled water.

0.5 M Triethanolamine - 0.05 M EDTA buffer (pH 7.6).

Triethanolamine hydrochloride	23.3 g
EDTA disodium salt	0.47 g
Distilled water	200 ml

The pH was adjusted to 7.6 with approximately 20 ml of 2 M sodium hydroxide and the final volume was made up to 250 ml with distilled water.

2.5. REAGENTS.

Tyrosine reagent.

Solution A :

0.1 % 1-nitroso-2-napthol in 95 % ethanol

Solution B :

Conc.nitric acid:distilled water	1:5	49.0 ml
2.5 % Sodium nitrite		1.0 ml

Solution A was added to an equal volume of solution B immediately prior to use.

CHAPTER 3: METHODS.

3.0.Transplantation of tumours in NMRI mice.

The MAC13 and MAC16 colon adenocarcinomas were originally induced with 1,2-dimethylhydrazine by Dr J.Double, Bradford University (Bibby et al, 1987). Of these two tumours only the MAC16 colon adenocarcinoma showed symptoms of cachexia in the host. The MAC16 adenocarcinoma was excised from donor animals, placed in sterile isotonic saline and cut into small fragments 1 x 2 mm in size. A trocar was used to implant fragments of the tumour subcutaneously into the flank of the right hind limb of NMRI mice (Mr. M. Wynter, Aston University). The doubling time of this tumour was determined to be 3-4 days by Mr M. Wynter, Aston University. The mice were weighed daily and were sacrificed if they had lost more than 30% of their original body weight, or if the tumour ulcerated, or if the animal became moribund. This was agreed by the Co-ordinating Committee on cancer research of the United Kingdom for the welfare of animals with neoplasms. Mice bearing the MAC16 tumour were deemed to be cachectic 14 to 16 days after transplantation when the tumours became palpable and a 2-4 g weight loss occured. However, not all mice bearing the MAC16 tumour lost weight, a small proportion (15-20%) maintained a constant weight and were consequently termed non-cachectic.

Fragments of the MAC13 adenocarcinoma, originally supplied

by Dr J. Double, Bradford University, were implanted in the flank of NMRI mice. This was found to have a doubling time of 2-3 days as determined by Mr M. Wynter, Aston University. There was no weight loss experienced during the growth of the tumour.

3.0.1. Collection of blood samples.

Between 9.30 and 10.30 am blood was removed from mice by cardiac puncture under anaesthesia using a mixture of halothane, oxygen and nitrous oxide. Approximately 1.0 ml of blood was collected from each mouse using a heparinised or un-heparinised syringe depending if whole blood, plasma or serum was required. The blood samples were transferred to microfuge tubes and kept on ice for plasma and left to clot for 10 min at room temperature for serum. The samples were then microfuged at 13000rpm for 5 min and the resultant plasma/serum was removed and frozen.

3.1. Body composition analysis.

The gastrocnemius and thigh muscle were carefully dissected out from the left leg of the carcass and weighed. The carcass and muscles were placed on pre-weighed tin foil in an oven at 80^{o}C until a constant weight was achieved. Dry weights of the carcass and muscles were separately recorded. The water content for the muscle and total

carcass was then calculated from the wet and dry weights. Total fat content of the carcass was determined using the method of Lundholm et al (1980). Each carcass was broken up into small pieces and ground with a pestle and mortar. The powder was then extracted in turn with 25 ml of acetone:ethanol (1:1 v/v), chloroform:methanol (1:1 v/v) and diethylether. The extracts were combined in a pre weighed round bottomed flask. The solvents were then removed under vacuum using a Buchi rotary evaporator to leave a fatty residue. The flask was reweighed and the total fat content per carcass calculated.

3.1.1. Kjeldahl nitrogen determination.

A microkjeldahl was used to measure the nitrogen content of the total mouse carcass, the gastrocnemius muscle and the liver. The principle of the method is as follows. Digestion with strong sulphuric acid in the presence of a catalyst converts the nirogen in the sample to ammonium sulphate. The ammonia is released by alkali and carried by steam-distillation into boric acid. The amount of ammonia is then estimated by titration with acid.

3.1.2. Preparation of samples.

The whole mouse carcass was homogenised, freeze dried and ground with a pestle and mortar to achieve a homogeneous

mixture. The gastrocnemius muscle and liver were analysed as a whole.

3.1.3. Analysis of samples.

500mg of ground mouse carcass or the entire gastrocnemius muscle and liver were added to a 50ml Kjeldahl flask. 5ml of concentrated sulphuric acid and 150mg of the catalyst mixture were carefully added. The catalyst mixture contained 32 parts potassium sulphate, 5 parts copper sulphate and 1 part selenium powder, ground fine and mixed. The flasks were heated strongly on an incineration rack for 30 min until the digest cleared. The samples were then quantitatively washed from the flasks and a 1ml sample was analysed. The ammonia in the sample was released by the addition of 5ml of 40% sodium hydroxide and carried by steam distillation into 10ml saturated boric acid solution. The absorbed ammonia was then titrated with 0.01M hydrochloric acid and the amount of nitrogen contained was calculated from the titration volume.

3.2. In vitro determination of the rate of protein synthesis and degradation in the MAC16 tumour-bearing mouse.

3.2.1. Preparation of isolated mouse gastrocnemius muscle.

Female NMRI mice were killed by cervical dislocation, their gastrocnemius muscles were quickly ligatured and dissected out and placed in ice-cold isotonic saline. The muscles were then blotted, weighed and carefully tied via the tendon ligatures to stainless steel incubation supports to prevent contraction thus improving protein balance and energy status.

3.2.2. Measurement of synthesis.

The muscles were placed in incubation vessels containing 3ml of Dulbecco's minimal essential media (DMEM) saturated with 95% O_2 : 5% CO_2 and were incubated for 30min at 37°C. After this pre-incubation the muscles were rinsed in non-radioactive media followed by replacement with fresh media containing 20μCi of L-[4-^{3}H]phenylalanine (sp.act. 46.3 mCi mmol^{-1}). The muscle preparation was gassed continuously whilst incubating for a further 2h. At the end of this period the muscles were rinsed in non-radioactive medium, blotted and sonicated in 3ml of 2% $HCLO_4$ as described in section 3.2.3.1.

3.2.3. Measurement of degradation.

Female NMRI mice were injected via the intraperitoneal route with 0.25ml of physiological saline containing 150uCi of L-[4-^{3}H] phenylalanine (1.5 Ci mmol^{-1}). After 24h the

mice were killed by cervical dislocation and their gastrocnemius muscles were isolated and pre-incubated as above. After rinsing the muscles were transferred to 3ml of DMEM and gassed continuously for the final 2h incubation. At the end of the incubation period the muscles and media were treated as in section 3.5.3.1.

3.2.3.1. Analysis of protein synthesis and degradation samples.

The muscles resulting from the initial incubations were treated as follows. The muscles were placed in 3ml of 2% $HClO_4$ and sonicated until homogeneously dispersed. The samples were then centrifuged at 2800g for 15 min and the supernatant was added to 1.5ml of saturated tripotassium citrate to give a pH close to 6.0. The insoluble potassium perchlorate was removed by centrifugation at 2800g for 15 min and 1ml of the supernatant was diluted (1:1) and added to 10ml Optiphase Hi-safe 3 scintillation fluid for the measurement of the intracellular free pool of L-[4-^{3}H] phenylalanine.

The precipitate from the original centrifugation was washed three times with 5 ml of 2% $HClO_4$ and hydrolysed in 5 ml 6M HCl at 110^{o}C in sealed glass tubes for 24 h. The hydrolysates were evaporated to dryness and the residue was dissolved in 10 ml distilled water. A 1ml sample of the

solution was counted for [^{3}H] phenylalanine radioactivity to give the protein bound radioactivity. The rate of protein synthesis was calculated by dividing the amount of protein bound radioactivity by the amount of acid soluble radioactivity. The rate of protein degradation was calculated by dividing the amount of [^{3}H] phenylalanine radioactivity released into the incubation media during the final 2 h incubation period by the specific radioactivity of protein bound [^{3}H] phenylalanine.
For further discussion see appendix.

3.2.4. The effect of eicosapentaenoic acid on protein synthesis and degradation.

This experiment was carried out in collaboration with Dr S.Beck, Aston University. Female mice (average body weight 20g) were transplanted with fragments of the MAC16 tumour as described in section 3.0. and were fed on rat and mouse breeding diet for 10 to 12 days after transplantation when the tumours became palpable and weight loss had started to occur. This point was chosen to ensure complete tumour take and weight loss prior to initiation of therapy. Animals were treated daily for 10 days with either solvent (liquid parafin : water, 2:1) or EPA at a dose of 2.0gkg^{-1}. After 10 days measurements of protein synthesis and degradation were carried out on these and a group of non-tumour bearing female NMRI mice according to the method in section 3.2.

3.3. Measurement of protein degradation using tyrosine release.

In this method tyrosine release was used as a measure of degradation. Tyrosine rapidly equilibrates between intracellular pools and the medium and it is neither synthesized nor degraded. The method used follows the isolated gastrocnemius muscle preparation and the incubation protocol of section 3.2.3. except the media used was Krebs-Henseleit bicarbonate buffer. After the final 2 h incubation 2ml of the buffer was removed, deproteinised with 200ul of ice cold 30% TCA and centrifuged at 2800 g for 10 min. The supernatants were used for the estimation of tyrosine by the method of Waalkes and Udenfriend (1957). 1 ml of 0.1% 1-nitroso-2-napthol in 95% ethanol and 1 ml of nitric acid reagent was added to the supernatants in glass centrifuge tubes. These were stoppered, shaken and placed in a water bath at 55^{o}C for 30 min. After cooling 5 ml of ethylene dichloride was added and the tube was shaken to extract the unchanged 1-nitroso-2-napthol reagent. The tubes were then centrifuged at 2800g for 10 min and the aqueous supernatant was transferred to a 4ml glass cuvette. The fluorescence of the tyrosine derivative resulting from its activation at 460nm was measured at 570nm on a Perkin-Elmer LS-5 luminescence spectrometer.

3.4. Measurement of guanidinobenzoatase activity.

This method of fluorimetric assay has been developed from that described by Coleman et al (1976). Guanidinobenzoatase cleaves the substrate 4-methylumbelliferyl-p-guanidinobenzoate (MeUmbGdnBzOH) at the guanidinobenzoate moiety to yield the fluorogenic product methylumbelliferone. A Perkin Elmer LS-5 luminescence spectrometer was used to establish the optimal excitation and emission wavelengths for the measurement of methylumbelliferone fluorescence in 0.1M sodium phosphate buffer pH6.0. The excitation and emission wavelengths were set to 323 and 446nm respectively.

A standard curve (0.1-10 nMoles) was constructed for methylumbelliferone dissolved in N-N-dimethylformamide. 5ul of the standard was added to a 4ml glass cuvette with magnetic stirrer and containing 2ml of 0.1M sodium phosphate buffer pH6.0. The resultant fluorescence was read in arbitrary units which was used as a measure of guanidinobenzoatase activity.

In the guanidinobenzoatase assay the first parameter to be established daily was the background level of fluorescence and the rate of non-enzymic base catalysed hydrolysis associated with the substrate MeUmbGdnBzOH. 5µl of 2mM substrate was added to the buffer (as above) and the base

rate and the rate of spontaneous methylumbelliferone cleavage per minute was established.

The basic assay consisted of 2ml of 0.1M sodium phosphate buffer pH6.0, 5µl 2mM MeUmbGdnBzOH and 5-100µl of sample containing guanidinobenzoatase. The amount of sample used was dependent upon the quantity of fluorescence produced.

3.4.1. Biological substances tested.

The following substances were assayed for guanidinobenzoatase activity.

Control NMRI mouse serum.
MAC16 non-cachectic mouse serum and tumour extract.
MAC16 cachectic mouse serum and tumour extract.
MAC13 mouse serum and tumour extract.
Control human serum.
Non-cachectic cancer patient serum.
Cachectic cancer patient serum.

3.4.2. Chromatographic techniques.

3.4.2.1. DEAE cellulose column chromatography.

Supernatants from crude tumour homogenates were fractionated by anion exchange chromatography using a DEAE

cellulose column eluting under a salt gradient. The DEAE cellulose column (dimensions 1.0 x 14.0cm) was equilibrated with 0.01M phosphate buffer (pH8.0). The material was eluted with a linear gradient of 0-0.2M NaCl in 0.01M phosphate buffer (pH8.0). The column was eluted at a flow rate of 15ml/h and the effluent from the column was collected in 1.0ml fractions. Tumour protein (1.3mg) was applied to the column. The level of guanidinobenzoatase activity was measured by the assay described in section 3.1.

3.4.3. Effect of other substances on guanidinobenzoatase.

The following substances were assayed for their effect on guanidinobenzoatase activity. IC50 values were established for each inhibitor.

	Conc$^{n.}$	Solvent.
Eicosapentaenoic acid.	0.1-12.8µM	Ethanol
		Equimolar lysine
		0.1M Na carbonate
Docosahexaenoic acid.	0.1-12.8µM	Ethanol
Arachidonic acid.	0.1-12.8µM	Ethanol
Linolenic acid.	0.1-12.8µM	Ethanol
Linoleic acid.	0.1-12.8µM	Ethanol
Oleic acid.	0.1-12.8µM	Ethanol
BZAR.	0.001-10nM	1mM Aspartic acid

DNS-GGACK.	0.01-100μM	Ethanol
Trypsin inhibitor.	5μl 1mg/ml	dH_2O
MAC16 tumour extract. (0.1g tumour/ml)	5-200μl	0.9% saline

3.4.4. The molecular modelling of the fatty acid inhibitors.

The possible correlation between fatty acid structure and the degree of guanidinobenzoatase inhibition was investigated using two different systems of computer modelling. The two modelling packages used were Chemx and $Charm_m$ / Quanta - both of which were based largely on nuclear magnetic resonance parameters. The major difference between the systems is that $Charm_m$ is primarily a molecular mechanics program using information derived from large proteins whereas Chemx has parameters drawn from a vast array of widely differing small molecules.

The basic hydrocarbon chains of the fatty acids were constructed and then the molecules were "minimised". The aim of minimisation was to find a set of coordinates representing a molecular conformation with minimum potential energy. Chemx minimises using a quadratic gradient algorithm which computes the energy gradient with respect to specified minmisation variables. The $Charm_m$ system was more complex - the first step was the

computation of a single energy point for the fatty acid. This was a summation of the internal coordinate terms and pairwise non-bonded interaction terms. The next step termed steepest descent, was basically to iron out any steric hinderance and improve a poor conformation produced during the drawing phase. The final minimisation step involved the Adopted-basis Newton-Raphson algorithm which performed energy minimisations on the previous set of coordinates until they were adjusted to the lowest possible potential energy. The molecular models produced were given added resolution by generating dot surfaces coloured on Van de Waals and electrostatic energies.

In an attempt to try to correlate a particular structural aspect of the fatty acid to activity the "mreg" computer package was used to compute regression analysis on single or multiple variables of the Chemx and $Charm_m$ parameters. The parameters used were electrostatic and true volume, width, length, molecular weight and number of double bonds in conjuction with the reciprocal of inhibitory activity.

3.4.5. Determination of the Michaelis constant for guanidinobenzoatase and the K_i values for EPA and BZAR.

The K_M value for guanidinobenzoatase was established using a variation of the above method - section 3.1. Control NMRI mouse serum was used as the source of the enzyme and a

range of MeUmbGdnBzOH concentrations (2-12μM) was used. For each substrate concentration the rate of reaction was determined over a 1 min period with and without the presence of EPA and BZAR (3.2μM). The K_M value is derived from a double-reciprocal Lineweaver Burke plot where the slope K_M/V_{max} intercepts the x-axis on $-1/K_M$. The K_i value is calculated from the above plot for each inhibitor using a substituted Michaelis Menten equation where :-

Slope = K_M/V_{max}.

+ Inhibitor Slope = K_M/V_{max} $(1 + I/K_i)$.

3.4.6. Measurement of trypsin activity.

Trypsin hydrolyses the chromogenic substrate N-a-benzoyl-L-arginine-7-amido-4-methylcoumarin to yield the fluorescent product 7-amino-4-methylcoumarin. 100μl of 0.1mM substrate was added to 2ml of 50mM Tris Hcl buffer pH8.0 containing 20mM calcium chloride and 1% DMSO. The addition of trypsin 5μl 10U/ml hydrolysed the substrate causing an increase in emission at 440nm that was measured at 380nm. The effect of the guanidinobenzoatase inhibitors EPA (0.1-12.8μM) and BZAR (0.001-10.0nM) was also tested.

3.4.7. Measurement of esterase activity.

5μl of the esterase substrate o-nitrophenol butyrate was

added to 10ml of 0.05M Tris HCl buffer pH8.0 containing 20mM calcium chloride and 1% DMSO to form a stock solution. The assay volume was made up from 1ml of stock, 1ml of buffer and 10µl of esterase. The absorbance of the samples was measured at 412nm on a Beckman spectrometer. The effect of the guanidinobenzoatase inhibitors EPA (0.1-12.8µM) and BZAR (0.001-10.0nM) was also tested.

3.4.8. Photometric determination of plasminogen activators.

The method used to assay plasminogen activators has been developed by Coleman and Green (1981). This uses thiobenzyl benzyloxycarbonyl-L-lysinate (Z-Lys-SBzl) as a substitute for the plasmin substrate in a two step coupled assay.

Step 1. Plasminogen via plasminogen activator forms plasmin.

Step 2. Z-Lys-SBzl and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reacts with plasmin to form Z-Lys, mixed disulphide and thiophenolate.(Emax=412nm)

In step 2. the plasmin formed in step 1. is quantified - further plasminogen activation is carefully minimised.

Step 1. reagents.

Glycine-BSA: 1.0M sodium glycinate and 5mg/ml BSA pH8.5,

stored frozen and diluted 5-fold prior to use.

BSA-HCL: 1.0mg/ml BSA in 1.0mM HCl titrated to pH3.0 by the addition of 1.0M HCl, stored at 4°C.

Plasminogen activator:

1.0 U/µl in BSA-HCl, stored frozen and serially diluted with BSA-HCl to stock solutions of $0.2\text{-}0.2\text{x}10^{-4}$ U/µl immediately prior to use.

Plasminogen: Stored frozen, weighed immediately prior to use and dissolved in BSA-HCl to 1mg/ml.

Step 2. reagents.

Triton X-100: 0.1% (w/v) in distilled water, stored at room temperature.

DTNB: 22mM DTNB in 50mM di sodium hydrogen phosphate, stored at 4°C.

Pi-NaCl: 200mM sodium phosphate plus 200mM NaCl pH7.5, stored at 4°C. (Phosphate saline)

STI: 1mg/ml soyabean trypsin inhibitor in 1.0mM HCl, titrated to pH3.0, stored at 4°C.

In step 1. the plasminogen activation takes place in an assay volume achieved by the addition of 40µl of glycine-BSA, 5µl of plasminogen activator (tissue-type plasminogen activator) and 5µl of plasminogen. The plasminogen is added last to the assay mixture already equilibrated at 37°C. The controls for the plasminogen

activator are an equal volume of BSA-HCl substituted into the assay. After 45 min step 1. is terminated by dilution with step 2. reagents.

The colour reagent (diluent) is made by the addition of 1 part of Triton X-100, DTNB and Z-Lys-SBzl stock solutions to 100 parts of Pi-NaCl at 37°C just prior to use. The addition of 950ul of this reagent to 50µl of step 1. initiates step 2. The colour reaction is allowed to proceed for 60min at 37°C - it is terminated by the addition of 100µl of the STI solution. The absorbance of the solution is measured immediately at 412nm on a Beckman spectrometer.

3.4.8.1. Effect of other compounds on tissue-type plasminogen activator.

The addition of these compounds to the assay was made by substituting 5µl of the 40µl BSA-Glycine in step 1.

	Conc^{n}.	Solvent
Eicosapentaenoic acid	0.1-12.8µM	0.1M Na carbonate Equimolar lysine
BZAR	0.001-10nM	1mM Aspartic acid

3.5. Characterisation of the differences between cell

bound and free guanidinobenzoatase.

3.5.1. Cell bound guanidinobenzoatase activity.

These experiments were carried out in collaboration with Dr. F.S.Steven, University of Manchester.

3.5.1.1. Preparation of tumour sections.

The freshly removed MAC16 solid tumour was placed in a 4% solution of formaldehyde in 0.9% isotonic saline to fix for a week. It was then dehydrated through a series of alcohols - 70%, 90%, 100% and 100% for 2h each. The tissue was then cleared in two changes of chloroform overnight before being infiltrated with wax. The wax infiltration required four 2h steps :-

1. Toluene 3 parts - wax 1 part
2. Toluene 1 part - wax 1 part
3. Toluene 1 part - wax 3 parts
4. Toluene 0 parts - wax 4 parts

The entire tumour was then embedded and block mounted in wax using a Shandon Histocentre. Sections (4μm) were cut on a Reichert - Jung Biocut 2030 microtome. The sections were then carefully floated onto an electrothermal mounting bath at 42°C. The warmed sections expanded to normal proportions before being floated onto pre-cleaned microscope slides. The sections were manoevered into

permanent positions, drained with blotting paper and dried completely on a warming plate. The sections were then dewaxed and rehydrated according to the following protocol:-

1.	Toluene	3 min
2.	Toluene	3 min
3.	50/50 Toluene, 100% alcohol	3 min
4.	100% alcohol	2 min
5.	100% alcohol	2 min
6.	70% alcohol	2 min
7.	50% alcohol	2 min
8.	30% alcohol	2 min
9.	15% alcohol	2 min

3.5.1.2. Pretreatment and staining of dewaxed sections.

The following pretreatment, staining and photography of fluorescence micrographs was carried out by Dr.F.S.Steven, University of Manchester. The basic staining technique involved the use of the fluorescent probe 9 amino-acridine (9AA)-binding to the active site of guanidinobenzoatase by competitive inhibition. The following protocols were used to determine the effect of EPA and mouse serum on cell bound guanidinobenzoatase.

1. The dry dewaxed control slides were stained in 1mM 9AA for 1-2 min before being washed for 1 min in 0.9% NaCl.
2. EPA was dissolved in methanol and diluted with

$1.5x10^{-4}$M leucine in phosphate buffered saline to give a concentration of 37µM. 10ul of the EPA was applied to the section - after 30 min 20µl of 9AA was added for a further 2 min. This procedure was followed by a 1 min wash in 0.9% NaCl.

3. These sections were treated with EPA as above for 30 min and were then placed in a 300ml tank of 0.1mM 9-aminoacridine for 1 h - the concentration of EPA in the staining bath was diluted sufficiently to be considered minimal. This procedure was followed by a 1 min wash in 0.9% NaCl.

4. 50µl of mouse serum was applied to the sections - after 1 h the sections were washed for 1 min in 0.9% NaCl and stained with 9AA according to protocol 1.

The 4 treatment groups were then viewed under a fluorescent microscope to determine the degree of 9AA staining.

3.5.1.3. Preparation, pretreatment and staining of frozen sections of MAC16 tumour.

The MAC16 tumour was frozen onto the cutting block of the freezing microtome with carbon dioxide and sections were then cut and mounted by Dr.W.Field, Aston University. These sections were used to try and demonstrate that cell bound guanidinobenzoatase is a distinct enzyme, dissimilar from the serum enzyme. The test sections were placed in a wet box for 1 h with 15µl of 0.9% NaCl applied to the

surface to solubilize guanidinobenzoatase inhibitors. The sections were then washed in 3 tanks of 0.9% NaCl for 1 h to remove cytoplasmic proteins. The control sections were washed as above. The control and test sections were then exposed to fibrin films to remove guanidinobenzoatase and guanidinobenzoatase-inhibitor complexes before being tested with 9AA.

3.6. Characterisation of the proteolytic factor.

The method in section 3.3. was used as the basis for characterising the proteolytic factor. Serum from MAC16 tumour-bearing animals with progressive weight loss was added to the assay as a source of proteolytic material and non-tumour bearing NMRI mouse serum was used as a control. 280ul of serum was added to each assay to give a final concentration of 7% serum per assay. The serum that was added to the assay constituted the volume of serum per mouse that would circulate around the musculature every few seconds. Serum from animals with 11-15% weight loss was used subsequently as the source of the proteolytic factor. For further discussion see appendix.

3.6.1. Effect of various treatments on tyrosine release from isolated gastrocnemius muscle.

The following were tested in vitro :

Non tumour-bearing mouse serum

MAC13 mouse serum

Cachectic MAC16 mouse serum

Heated 60°C/5min cachectic MAC16 mouse serum

Partially pure lipolytic factor isolated from MAC16 tumour

Cachectic MAC16 mouse serum + 200μM indomethacin

Cachectic MAC16 mouse serum + 1mM PMSF

Cachectic MAC16 mouse serum + 1.77mM BW4AC

Cachectic MAC16 mouse serum + 500μM EPA

3.6.2. In vitro determination of the role of prostaglandin E_2 in isolated gastrocnemius muscle proteolysis.

3.6.2.1. Preparation of samples.

The gastrocnemius muscles were removed, weighed, and sliced on filter paper moistened with 0.85% sodium chloride. The slices were incubated in 2ml Krebs-Ringer bicarbonate buffer supplemented with 1mg/ml glucose and BSA in a shaking water bath at 37°C. The initial incubation was for 20 min in a gas phase of 5% CO_2: 95% N_2 and then for a further 15 min in a gas phase of 5% CO_2: 95% O_2. At the end of the incubation period 1ml of the surrounding buffer was removed and adjusted to pH3 with 2M HCl. This was then extracted twice with 3ml of ethyl acetate saturated with water and evaporated to dryness under a stream of N_2. The

residue was then dissolved in 1ml of 0.025M phosphate buffer, pH6.8, containing 0.01M EDTA, 0.9% NaCl, 0.3% bovine gamma-globulin, 0.005% triton X-100 and 0.05% sodium azide.

3.6.2.2. Determination of prostaglandin E_2 levels by radioimmunoassay.

A stock solution of 1ug/ml PGE_2 was prepared in absolute ethanol. This was diluted in 0.01M sodium phosphate buffer (pH7.4) containing 0.15M NaCl, 0.1% BSA and 0.1% sodium azide to give standard concentrations of 0 - 1000pg/0.1ml. A 1ml aliquot of sample or standard was added to 0.5ml of reconstituted rabbit anti-prostaglandin E_2 antiserum in polypropylene test tubes. The samples were mixed well and incubated at $4^{o}C$ for 30 min. [5,6,8,11,12,14,15-$^{3}H(N)$]-PGE_2 (0.1ml) was added to the samples to give a concentration of 4.26nCi per assay. The samples were mixed well and incubated for 60min at $4^{o}C$. A dextran coated charcoal suspension was prepared in sodium phosphate buffer 0.01M, pH7.4 (as above) containing 1.0% activated, untreated charcoal powder (250-350 mesh) and 0.1% dextran (approximate molecular weight 70000). It was important to ensure that the dextran was in solution before the addition of the charcoal and that the dextran coated charcoal suspension was stirred in ice for at least 30 min before and during use. The dextran coated charcoal suspension (0.2ml) was added to the samples

which were mixed thoroughly and incubated for a further 10 min on ice. The samples were then centrifuged in a Heraeus minifuge T at 2000g for 15min at 4°C. The clear supernatants were removed, added to 10ml of a xylene based scintillant Optiphase Hi-safe 3, mixed on a whirlimixer and the amount of radioactivity present was determined on a Packard Tri-Carb 2000A liquid scintillation analyser.

3.6.2.3. Effect of serum from non tumour-bearing and MAC16 tumour-bearing animals on isolated gastrocnemius muscle PGE_2 content.

Isolated gastrocnemius muscles were incubated as in section 3.3. 7% serum from non tumour-bearing and cachectic MAC16 tumour-bearing animals containing 147 ± 8pg/ml and 111 ± 7pg/ml PGE_2 respectively was added to the 4ml assay volume for the final 2h incubation. The muscles were removed from the incubation supports and were analysed for PGE_2 content according to section 3.6.2.

3.6.2.4. Effect of indomethacin on the PGE_2 content of and tyrosine release from isolated gastrocnemius muscle.

Indomethacin (50 - 200μM) and 7% serum from animals with 11-15% weight loss was added to the isolated gastrocnemius muscle preparation for the final 2h incubation (section

3.3.). The muscle PGE_2 content was measured using the radioimmunoassay (section 3.6.2) and the levels of tyrosine release were measured concomitantly (section 3.3.).

3.6.2.5. Effect of prostaglandin precursors on tyrosine release from isolated gastrocnemius muscle.

Arachidonic acid and linoleic acid (0.01-100μM) and the triglycerides triarachidonin and trilinolein (5-50μM) were added to control serum in an attempt to mimic the effect of cachectic serum. Tyrosine release was measured.

3.6.2.6. Effect of in vitro and in vivo EPA treatment on the PGE_2 content of and tyrosine release from isolated gastrocnemius muscle.

EPA (100-500μM) and 7% serum from animals with 11-15% weight loss was added to isolated gastrocnemius muscle preparations from non tumour-bearing animals for the final 2h incubation (section 3.3.). Muscle PGE_2 content and tyrosine release were measured concomitantly (section 3.6.2., 3.3.). The effect of DHA (100-500μM) was compared to the effect of EPA.

Gastrocnemius muscles were isolated from MAC16 tumour-bearing animals that had been treated with EPA (section 3.3.4.). These were compared with muscles from

untreated non tumour-bearing animals. Serum from non tumour-bearing animals and MAC16 tumour-bearing animals with 11-15% weight loss was added to the assay and muscle PGE_2 content and tyrosine release were measured.

3.6.2.7. Effect of serum from cachectic MAC16 tumour-bearing animals on protein synthesis.

Serum from animals with 11-15% weight loss was added to isolated gastrocnemius muscle preparations and the rate of protein synthesis was determined according to the protocol in section 3.2.

3.7. Branched-chain amino acid treatment.

Ten to twelve week old female NMRI mice weighing between 20 - 22g were transplanted with the MAC16 colon adenocarcinoma. The mice were fed a rat and mouse breeding diet and water ad libitum. Treatment was initiated 14 days after implantation when the tumour was palpable and cachexia became apparent (weight loss 6-8%). During treatment the branched-chain amino acids leucine 23.24mM, isoleucine 14.53mM, and valine 22.76mM were administered in the drinking water. The food and treated water were available ad libitum throughout the 5 day course. Four groups of mice were used :-

1. Non-treated cachectic MAC16.

2. Treated cachectic MAC16.

3. Non-treated NMRI.

4. Treated NMRI.

Throughout the treatment phase tumour volume, weight loss and food and water intake were measured. After 5 days blood was removed by cardiac puncture and the levels of plasma glucose, albumin, lactate and pyruvate were determined. The carcass and tumour were weighed prior to body composition analysis.

3.7.1. Determination of blood glucose levels.

Glucose levels were determined on whole heparinised blood using a Sigma diagnostic kit (no. 635). This utilises the methods of Hyvarinen and Nikkila (1962), and Feteris (1965) which involves the colourimetric measurement of a proportional blue-green complex formed between glucose and o-toluidine. Blood (100µl) was deproteinised with 0.9ml of 3% trichloroacetic acid and centrifuged at 3000g for 10 min and 0.3 ml of the supernatant was added to 3 ml of the o-toluidine reagent. The samples were boiled for 15 min, cooled and read on a Beckman DU70 spectrophotometer at 635nm.

3.7.2. Analysis of plasma albumin levels.

Albumin levels were determined on plasma using a Sigma

diagnostic kit (no. 631). This utilises a modification of the method of Doumas (1971) - albumin binds to bromcresol green (BCG) to produce a blue-green colour with an absorbance maximum at 628 nm. The intensity of the colour produced is directly proportional to the albumin concentration in the sample.

3.7.3. Analysis of blood lactate levels.

The levels of blood lactate were measured using the method of Hohorst (1957). Lactate is oxidised to pyruvate by NAD in the enzymatic reaction catalysed by lactate dehydrogenase.

$$\text{L-(+)-Lactate} + NAD^+ \xleftrightarrow{\text{LDH}} \text{pyruvate} + NADH + H^+$$

The formation of NADH is measured by the increase in extinction at 340 nm. The assay cuvettes contained 0.43 M glycine/ 0.34 M hydrazine buffer (pH 9.0), 2.75 mM NAD and 200µl of deproteinised sample in a total volume of 2.9 ml. The reaction was initiated by the addition of 20µl of lactate dehydrogenase (19 U/ml) and the increase in absorbance was measured for 60 min at a temperature of 25°C.

3.7.4. Determination of blood pyruvate levels.

The levels of pyruvate in the blood were determined using the reverse of the reaction shown in section 3.7.4.

(Hohorst, 1957). In this case the oxidation of NADH is proportional to the substrate converted and is measured spectrophotometrically at 340 nm. The assay cuvettes contained 300mM triethanolamine/3mM EDTA buffer (pH 7.6), 0.1mM NADH and 500µl of deproteinised sample in a total volume of 2 ml. The reaction was initiated by the addition of 20µl of lactate dehydrogenase (3 U/ml). The decrease in absorbance was measured for 20 min at a temperature of 25°C.

CHAPTER 4: RESULTS AND DISCUSSION.

4.0. Characterisation of the rates of protein synthesis and degradation in the female MAC16 tumour bearing mouse - the relationship to body composition and nitrogen content and the anticachectic effect of eicosapentaenoic acid.

4.0.1. Introduction.

Weight loss often preceeds clinical diagnosis in the cancer patient and this weight loss is of considerable prognostic value - the greater the weight loss, the poorer the prognosis and the lower the response rate to chemotherapy. (Shamberger et al, 1984). However, it is visceral protein and lean body mass depletion (as assessed by serum albumin concentration and creatinine : height index) that have in particular the worse prognostic impact than adipose depletion (Nixon et al, 1980).

The host finds itself at a distinct disadvantage sharing its resources with a tumour that is metabolically more versatile and aggressive. The energy requirement of the tumour is drained from the host as a consequence of metabolic necessity in a number of ways. The tumour acts as a nitrogen trap actively sequestering amino acids from the host plasma pool - this contributes towards a negative nitrogen balance in the host and a reciprocal nitrogen increase in the tumour. In addition the tumour has a vast requirement for host glucose - the glucose is metabolised

to lactate via the energetically inefficient Cori cycle, the lactate together with glycerol and alanine are reconverted to glucose via hepatic gluconeogenesis in an attempt to maintain host glucose homeostasis. The futile recycling of metabolic products results in net energy expenditure and this together with circulating catabolic factors produced by the tumour and tumour induced alterations in host metabolism result in the metabolic chaos associated with cancer cachexia. This is discussed in detail in section 1.0. Thus protein catabolism ensues. There is much controversy as to the relative merits of the roles played by protein syntheis and degradation in protein catabolism. Is the negative nitrogen balance often associated with the cancer patient due to depressed protein synthesis, increased degradation or a combination of the two ? Emery et al (1984) demonstrated in mice bearing the XK1 tumour that protein synthesis was depressed by 70% in muscle and 40% in liver and this reduction in the rate of protein synthesis could not be explained by a depression in food intake alone. Similarly Rennie et al (1983) support the view that muscle mass is regulated primarily by alterations in the protein synthetic rate and that changes in muscle degradation are largely secondary and adaptive. Conversely Tessitore et al (1987) attributed the marked weight loss seen in animals bearing a fast growing ascites hepatoma (Yoshida AH-130) to an elevation in the rate of muscle protein degradation with no apparent changes in

protein synthesis as measured by [^{14}C] bicarbonate labelling.

Kien and Camitta (1983) used a [^{15}N] glycine turnover technique to assess the rates of whole body protein turnover in sick children with newly diagnosed leukaemia or lymphoma. They found a significant increase in the childrens whole body protein turnover. Similarly Jeevanandam et al (1984) demonstrated that the rate of whole body protein turnover in cancer patients was about 30% higher than in non-cancer patients and starved normal subjects . Increased muscle proteolysis in weight losing cancer patients is also suggested by an increased venous excess of alanine from forearm muscles (Levin et al, 1983).

However, the mechanisms controlling protein synthesis and degradation are still largely unknown. The aim of this section was to establish the effect of progressive cachexia on skeletal muscle protein synthesis and degradation in a murine model of cancer cachexia (MAC16 adenocarcinoma). An attempt was made to correlate the changes in muscle turnover to alterations in host body compartments and nitrogen levels in the whole body, tumour, liver and gastrocnemius muscle. In addition the polyunsaturated fatty acid eicosapentaenoic acid (EPA) has been shown to effectively reverse the cachexia associated with the MAC16 tumour-bearing animal - consequently the possibility that

the anticachectic effect was mediated through a modulation of protein turnover was explored.

4.0.2. Results.

The main feature associated with the progressive cachexia seen in the MAC16 tumour-bearing animal is a significant decrease in carcass fat content - the body compartments represented as water and non-fat mass change correspondingly in proportion to total body weight (Fig.8). There is no significant reduction in the non-fat mass body component - this gives an estimation of total body protein. A microkjeldahl method was used to measure more accurately mouse carcass, tumour, liver and gastrocnemius nitrogen content. There is a significant decrease in whole body nitrogen content (Fig.9) between 16-30% weight loss expressed per g dry body weight. The nitrogen content of the tumour (Fig.10) increases dramatically at 6-10% weight loss and this increase is enhanced in proportion to tumour growth throughout the progression of cachexia - this contrasts with changes in mouse carcass nitrogen. There is a significant nitrogen loss seen in the gastrocnemius muscle (Fig.11) between 11-30% weight loss and this parallels the decrease in muscle wet weight (Table 1). There is also a significant decrease in the nitrogen content of the liver above 11% weight loss (Fig.12).

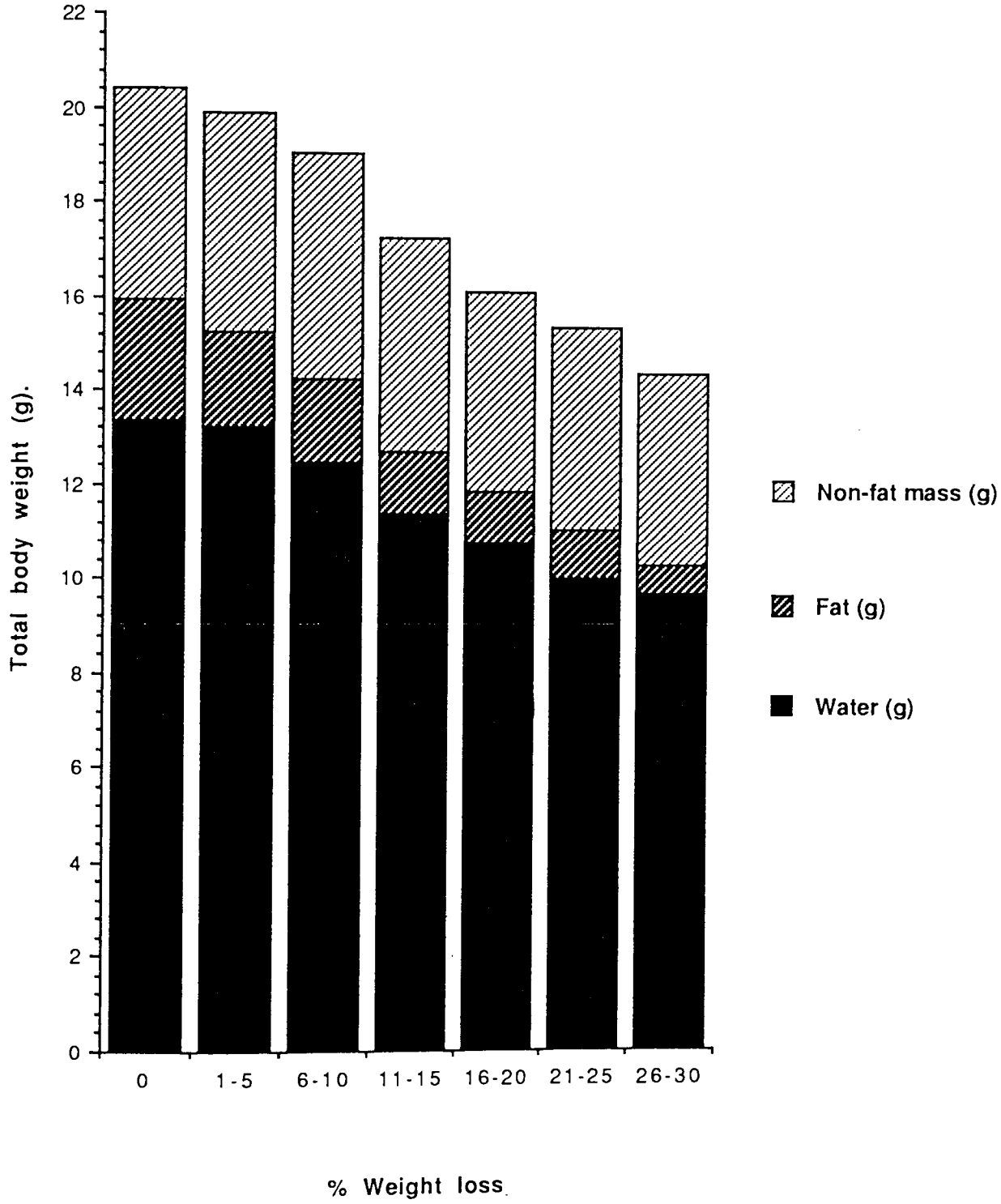
Table 1.

% Weight loss.	Gastrocnemius weight (mg) $\bar{X} \pm$ SEM.	P value v 0.
0	139.0 ± 2.4	-
1-5	131.7 ± 2.8	-
6-10	128.8 ± 3.3	-
11-15	126.0 ± 4.5	-
16-20	116.7 ± 6.8	P<0.05
21-25	106.9 ± 2.1	P<0.01
26-30	106.1 ± 2.9	P<0.01

Both synthesis and degradation of proteins were measured in isolated gastrocnemius muscle using L-[4-^{3}H] phenylalanine to label proteins. The results presented in fig.13 show that protein synthesis is significantly depressed in animals with a weight loss of between 16-20% and remains at a low level up to a weight loss of 30%. This is accompanied by an increased protein degradation, which increases with weight loss between 15-30% up to a maximum 240% increase at 30% weight loss. The non tumour-bearing animal exhibits the feature of growth in a young animal i.e. enhanced protein synthesis versus degradation. The oral administration of EPA 2.0gkg^{-1}/day to cachectic MAC16 tumour-bearing animals caused a 60% diminution in protein degradation when compared to a group of untreated cachectic mice and a 16% increase in protein synthesis which was not

Figure 8.

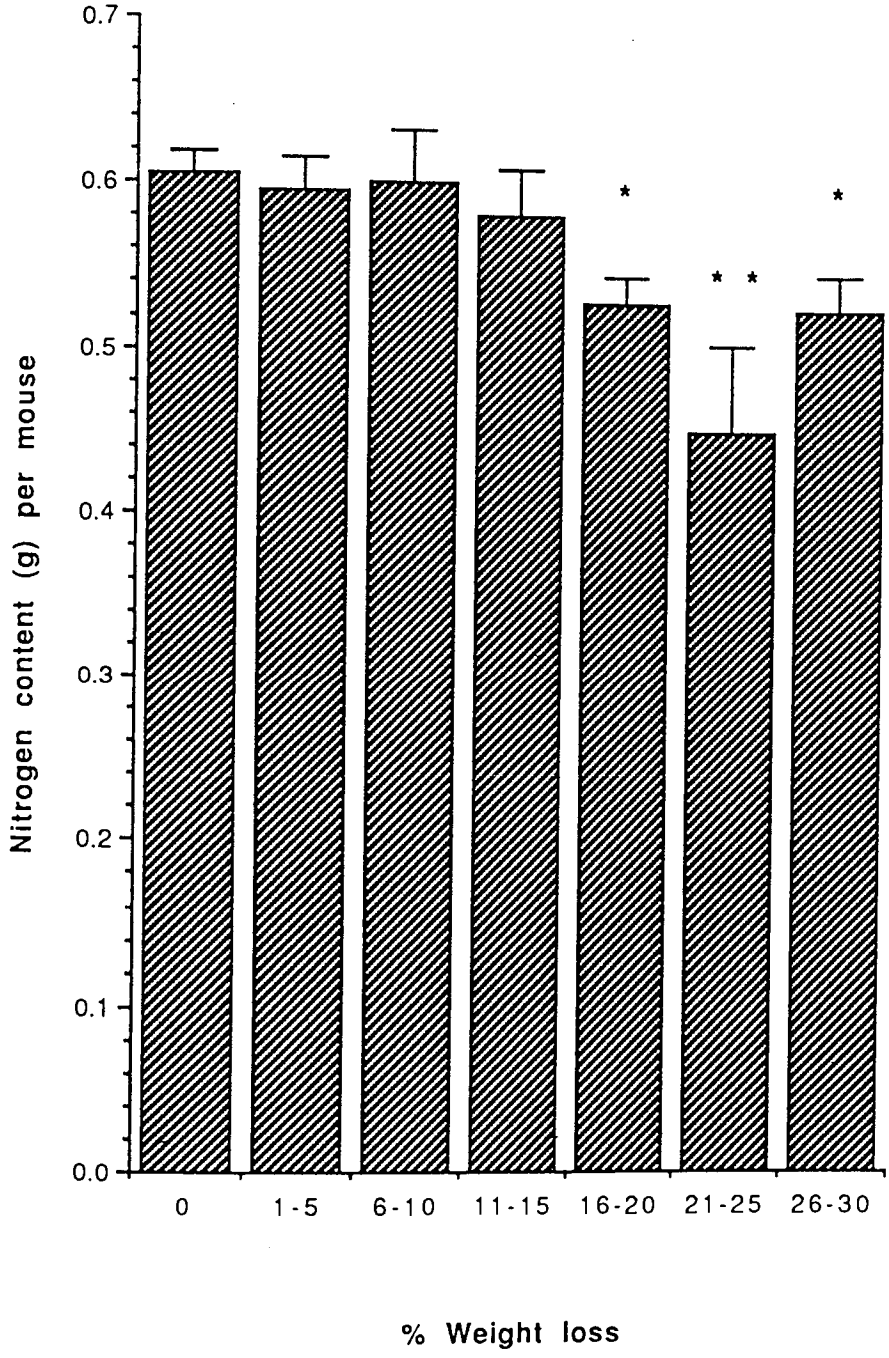
The effect of progressive cachexia on female mouse body composition.



Each bar represents the mean of 4 mice.

Figure 9.

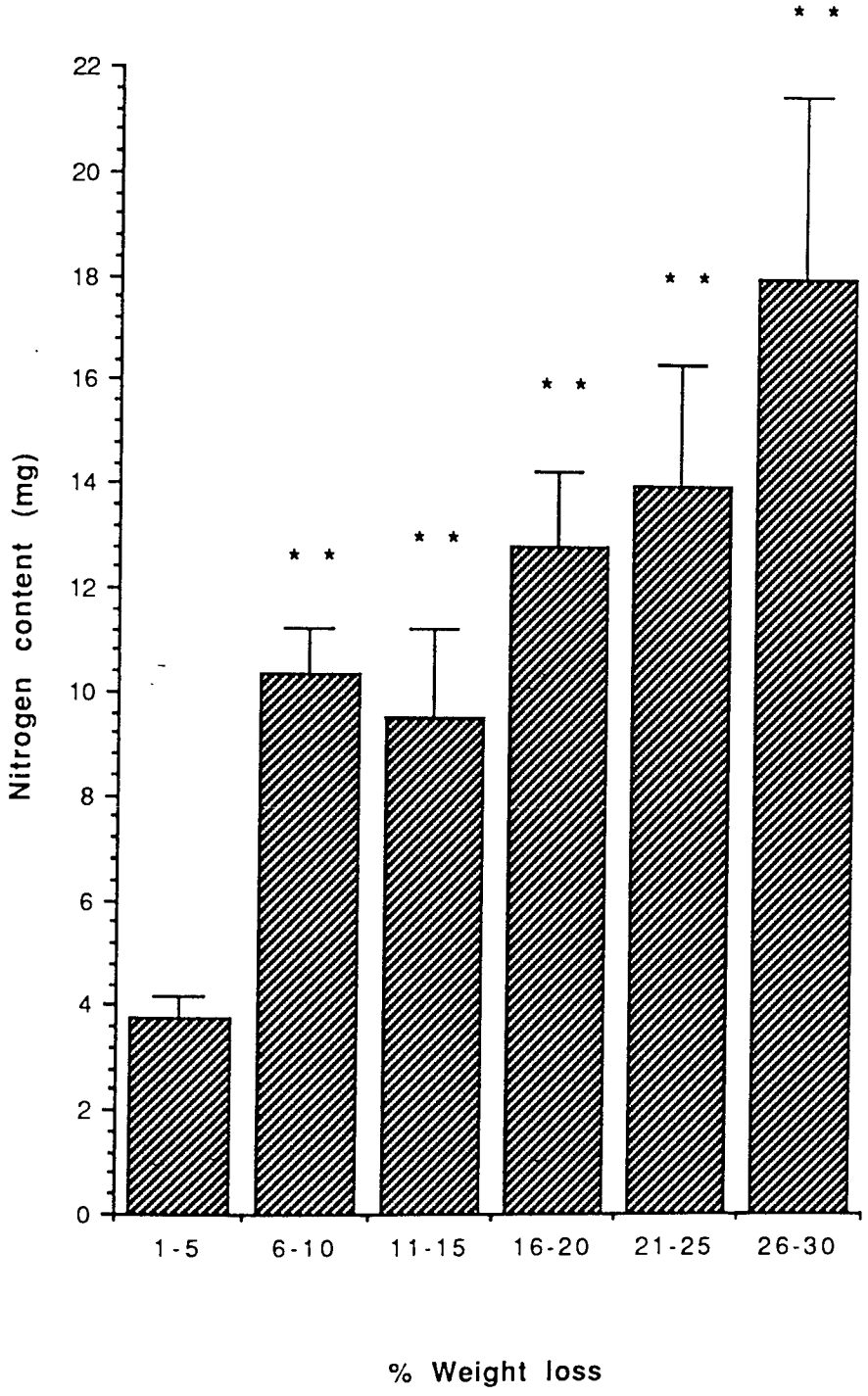
The effect of progressive cachexia on mouse carcass nitrogen content.



Each bar represents the mean ± SEM of 4 animals. Differences were determined by one-way ANOVAR as *=P<0.05, **=P<0.01 compared with non tumour-bearing animals (group 0).

Figure 10.

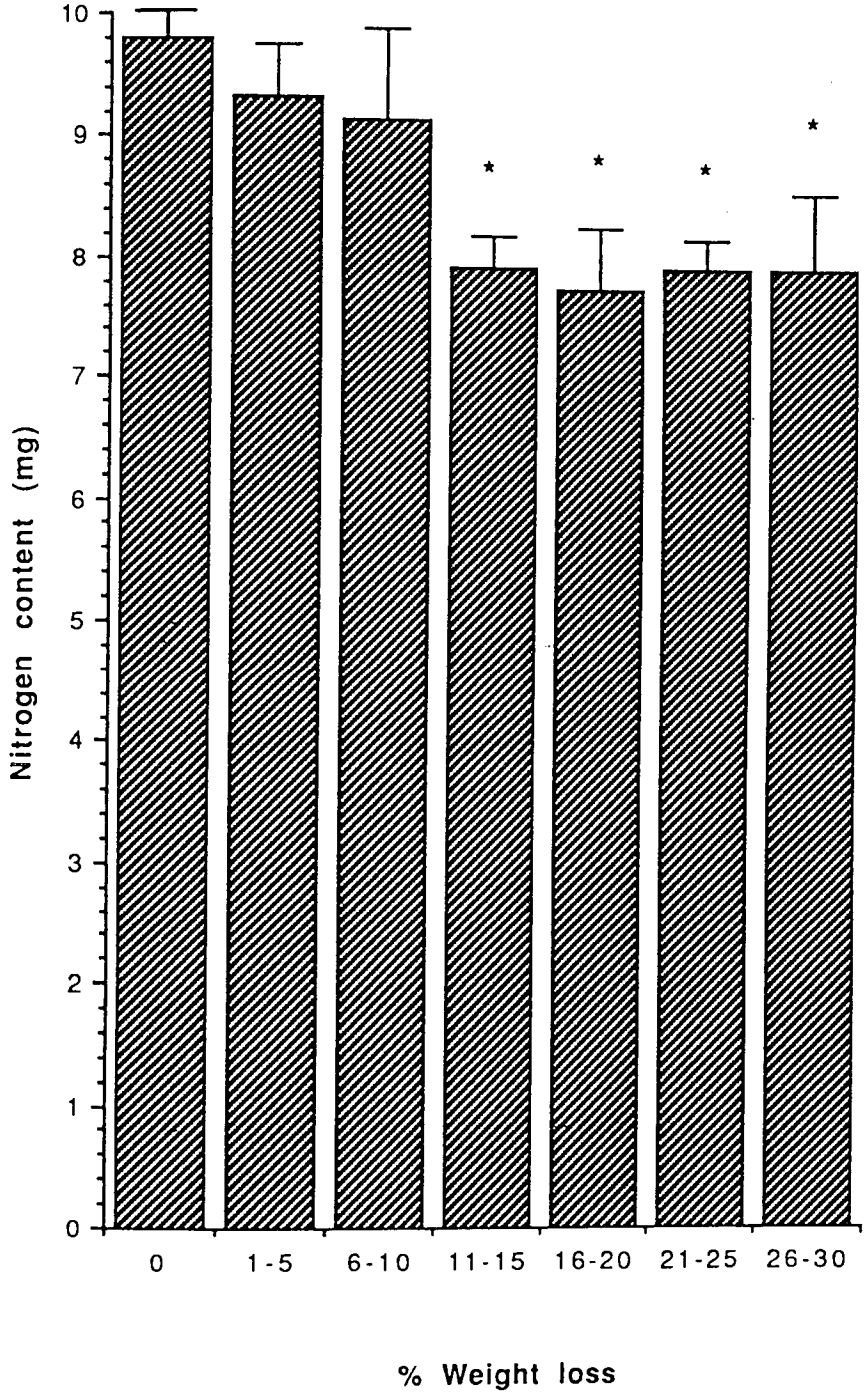
The effect of progressive cachexia on tumour nitrogen content.



Each bar represents the mean ± SEM of 4 animals. Differences were determined by one-way ANOVAR as **=P<0.01 compared with animals with 1-5% weight loss.

Figure 11.

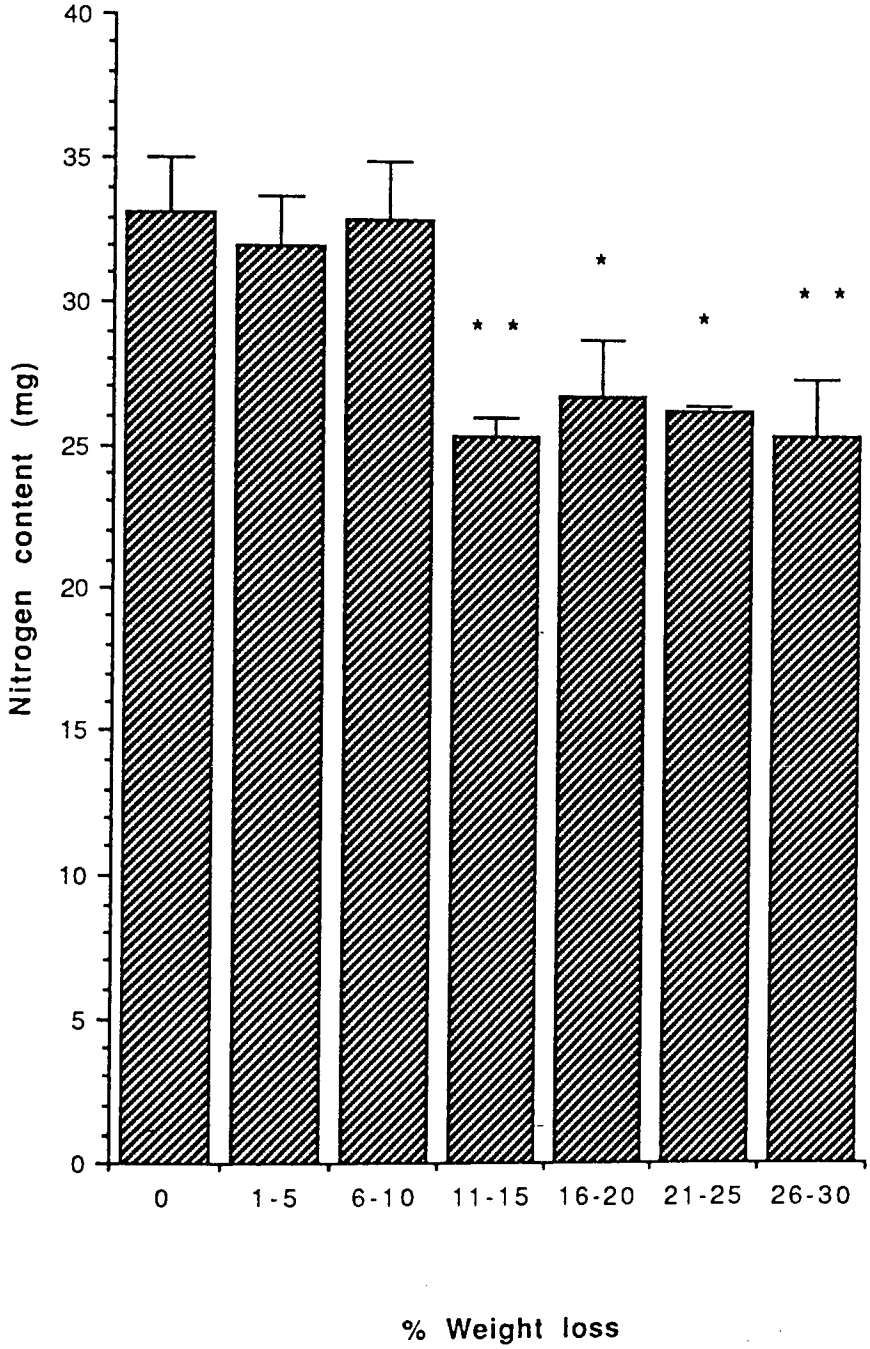
The effect of progressive cachexia on gastrocnemius nitrogen content.



Each bar represents the mean ± SEM of 4 animals. Differences were determined by one-way ANOVAR as *=P<0.05 compared with non tumour-bearing animals (group 0).

Figure 12.

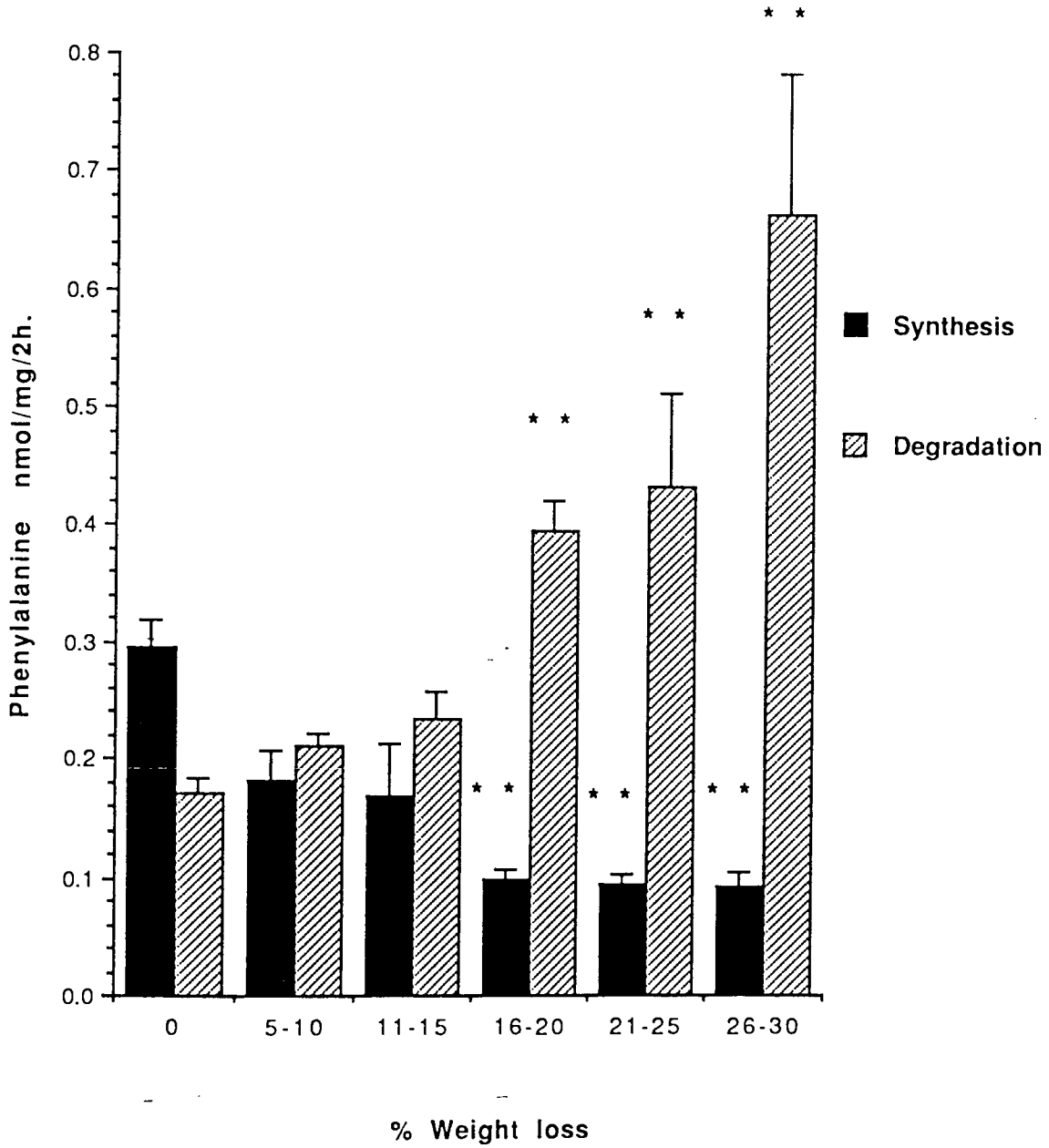
The effect of progressive cachexia on liver nitrogen content.



Each bar represents the mean ± SEM of 4 animals. Differences were determined by one-way ANOVAR as *=P<0.05, **=P<0.01 compared with non tumour-bearing animals (group 0).

Figure 13.

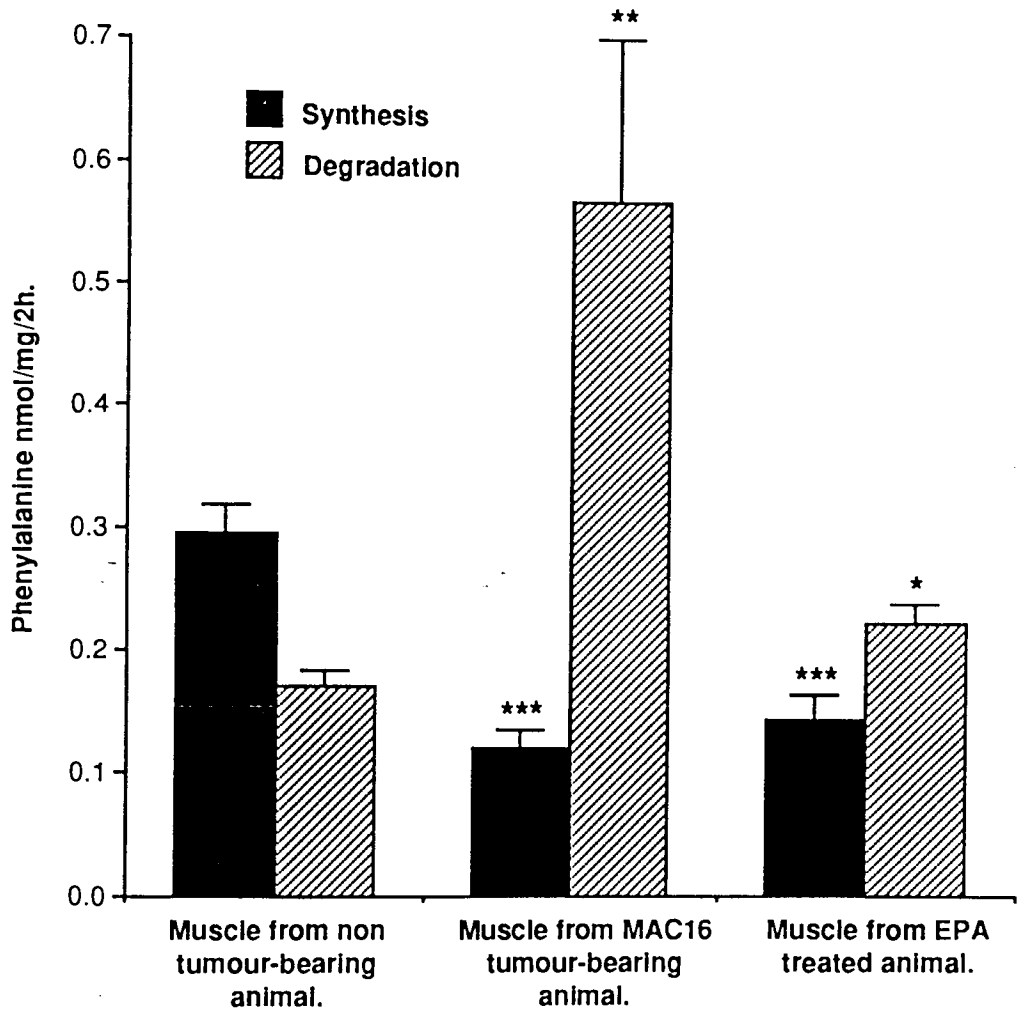
The effect of progressive cachexia on gastrocnemius muscle protein synthesis and degradation.



Each bar represents the mean ± SEM of 6 animals per group. Differences were determined by one-way ANOVAR as **=P<0.01 compared with non tumour-bearing animals (group 0). Protein degradation in animals with 26-30% weight loss is significantly different (P<0.05) from animals with 16-20% weight loss.

Figure 14.

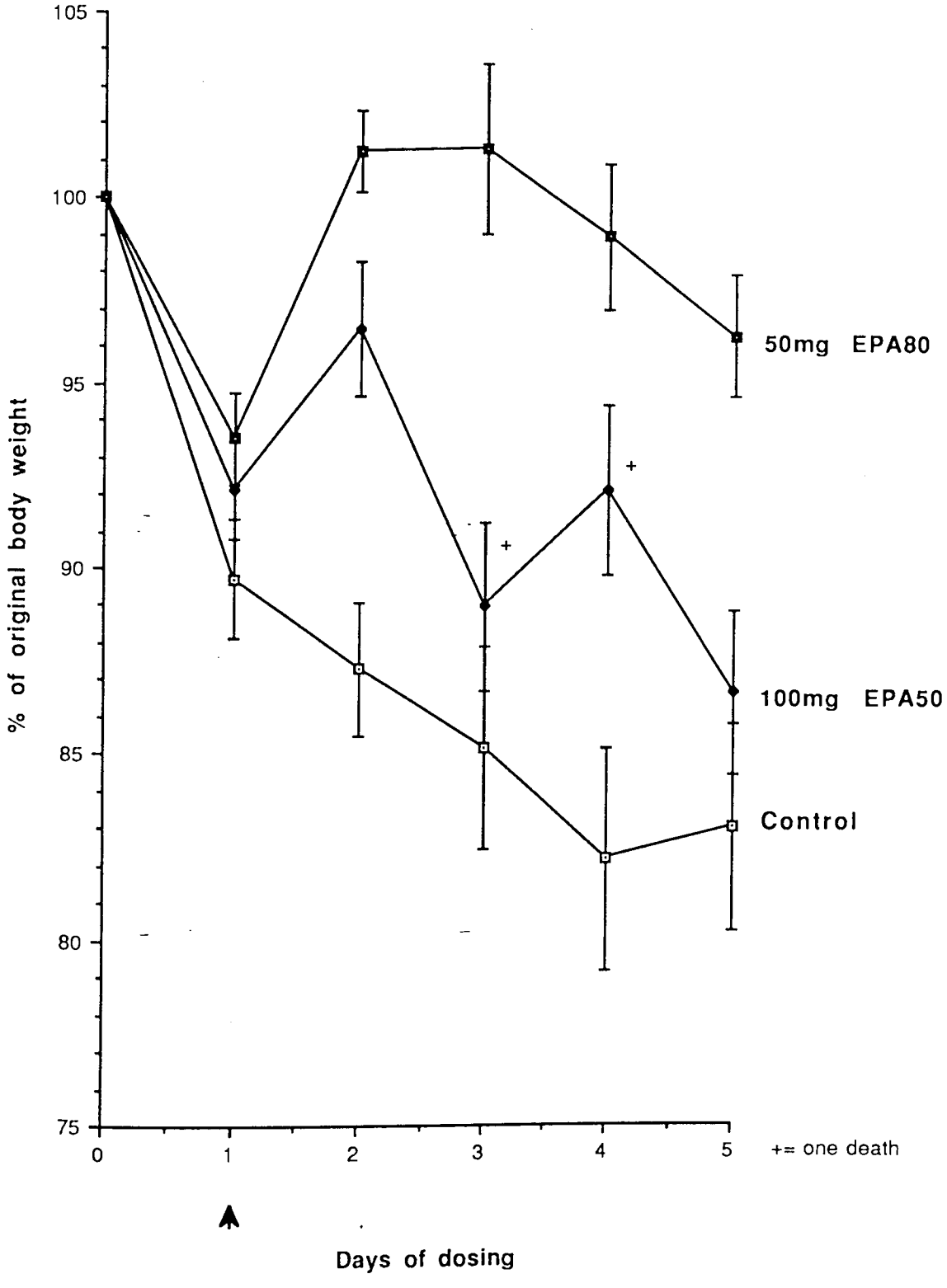
The effect of treatment of animals with EPA on gastrocnemius muscle protein synthesis and degradation.



Each bar represents the mean ± SEM for 6 animals per group. Differences were determined by Students T-test as **=P<0.01, ***=P<0.001 from non tumour-bearing animals and *=P<0.05 from MAC16 tumour-bearing animals.

Figure 15.

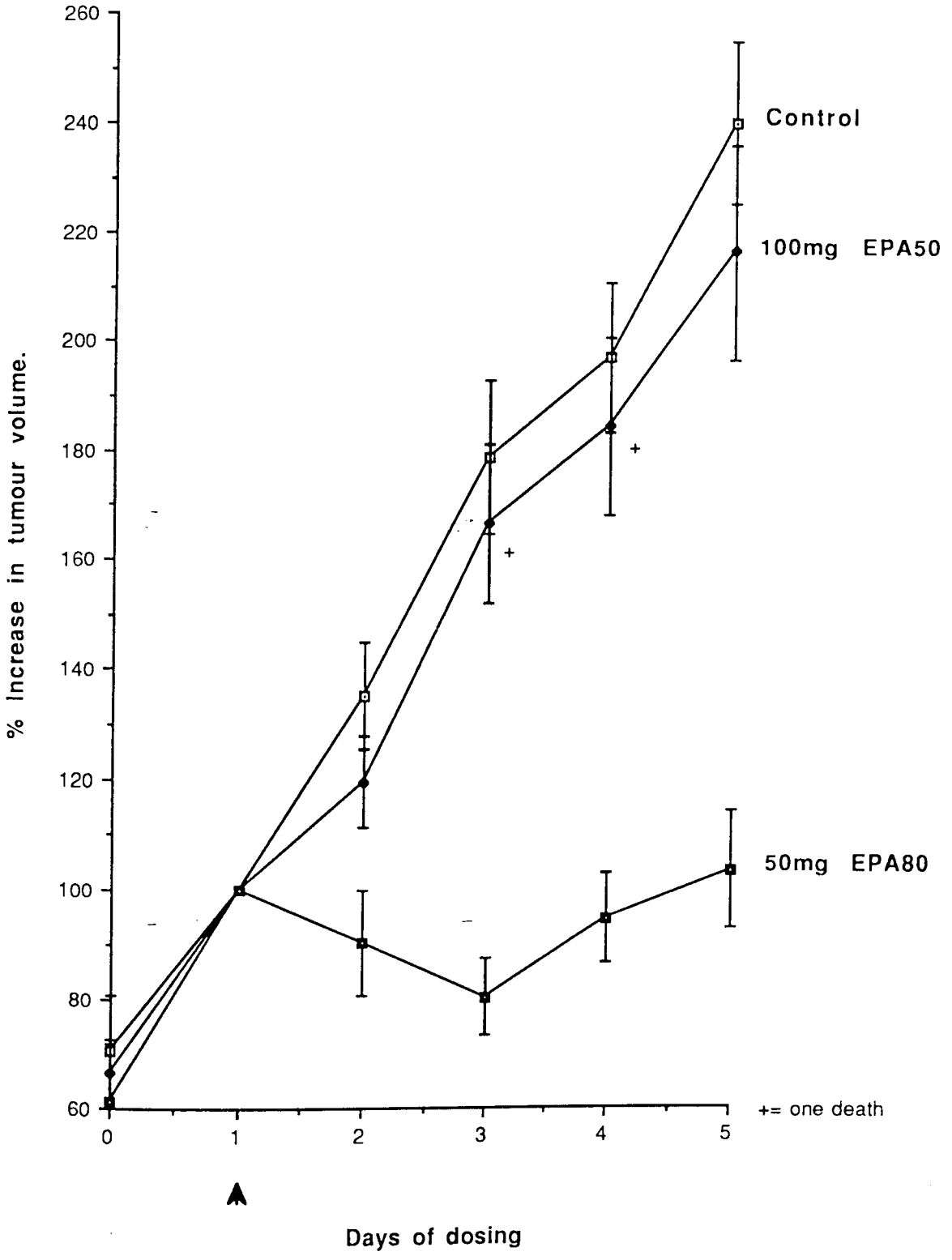
The acute effect of various doses of EPA on weight loss produced by the MAC16 adenocarcinoma.



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

Figure 16.

The acute effect of various doses of EPA on the growth of the MAC16 adenocarcinoma.



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

significant (Fig.14). This resulted in an approximate doubling of the animals life span through maintenance of body weight and a significant inhibition of tumour growth (fig. 15, 16).

4.0.3. Discussion.

From these results it was concluded that the MAC16 tumour partially mediates its influence on host metabolism by inhibiting protein synthesis and stimulating protein degradation. The loss of muscle mass that would be indicated by the massive increase in protein degradation is seen to some extent as the decrease in gastrocnemius muscle nitrogen content. However, this muscle accounts for only a very small percentage of the total skeletal muscle and it may be that the gastrocnemius muscle does not give a true representation of all skeletal muscle. Skeletal muscle is composed of a mixture of red and white fibres. Red or tonic fibres are rich in myoglobin, are specialised for oxidative phosphorylation, give a slow twitch response to stimulation, are more resistant to fatigue and are more efficient for generating sustained force. White or phasic fibres are specialised for anaerobic glycolysis, give a fast twitch response, fatigue more easily and are more efficient for quick intermittent movements (Alberts et al, 1983). Gastrocnemius muscle is composed mainly of white phasic fibres and Clark and Goodlad (1970) have

demonstrated an inherent suseptibility of these fibres and in particular the proteins of the contractile system to be influenced by the cachectic effect of the tumour. Consequently the whole body rate of protein degradation may be considerably modulated by the influence of the more resistant tonic fibres. Total body nitrogen begins to significantly diminish at 16-20% weight loss, reaches a minimum at 21-25% weight loss and then further nitrogen loss is prevented. The period of maximal nitrogen loss may be due to the increase in nitrogen excretion documented by Beck et al (1988) who demonstrated that male mice bearing the MAC16 tumour excreted nitrogen until they lost approximately 12-16% body weight and then a protein conservatory mechanism came into play and nitrogen balance was resumed. The loss of liver nitrogen seen in the MAC16 tumour-bearing animal throughout progressive cachexia can also be recognised in rats bearing the fast-growing ascites hepatoma (Yoshida AH-130)(Tessitore et al, 1987). However, in the case of the MAC16 tumour-bearing animal, the livers transient hyperplastic response is not discerible but the period of waste and regression attributable to an accelerated rate of protein degradation is a prominent feature.

Maintenance of host body weight in animals bearing the MAC16 tumour and treated with EPA is associated with decreased skeletal muscle protein degradation and a small

but insignificant increase in protein synthesis. The inhibitory effect of EPA on muscle protein degradation may result from its ability to inhibit prostaglandin E_2 synthesis - this is discussed further in section 4.6. However, a previous study by Palmer and Wahle (1987) has shown EPA to have no effect on protein synthesis or degradation in rabbit forelimb extensor muscles in vitro. The reason for this difference between the two studies is not known.

In conclusion it would appear that the MAC16 tumour increasingly gains nitrogen throughout the progression of cachexia at the expense of the hosts tissues - for example gastrocnemius and liver nitrogen - this is emphasized by the overall loss of carcass nitrogen. However, the gain of nitrogen by the tumour is insufficient to account entirely for the loss of carcass nitrogen - this suggests that the deficit is excreted. The pattern of nitrogen excretion in the female MAC16 tumour-bearing mouse throughout the progression of cachexia has yet to be fully elucidated. The loss of carcass nitrogen may be mediated by tumoural intervention increasing protein degradation and inhibiting protein synthesis. For further discussion see appendix.

4.1. Identification of the potential circulating mediator of muscle proteolysis associated with MAC16 colon adenocarcinoma induced cachexia.

4.1.1. Introduction.

Several factors have been postulated to account for an increased lipid mobilisation in the tumour-bearing state. These can be divided into direct lipid mobilising factors such as toxohormone L (Masuno et al, 1981), a serum factor produced by a thymic lymphoma in AKR mice (Kitada et al, 1982) and a serum factor produced by a cachexia inducing murine colonic tumour (MAC16)(Beck and Tisdale, 1987), and indirect factors such as tumour necrosis factor (TNF), which is thought to stimulate breakdown of adipose tissue as a result of the inhibition of the enzyme lipoprotein lipase, thus blocking synthesis of triglycerides (Oliff,1988). Similarly potential agents have been proposed to account for the lean body tissue depletion seen in cachexia. However, these factors are far less well characterised. A possible mechanism of direct action would be the systemic release by the tumour of enzymes capable of either direct extensive proteolysis or the initiation of a cascade of events leading to proteolysis, but with the abililty to escape or mute acute phase surveillance. The appearance of a tumour related enzyme in the serum could be the result of at least three processes: 1. Production by the tumour only of specific enzymes which are then released into the body fluids. 2. "Leakage" of enzymes from injured non-neoplastic cells. 3. "Induction" of host

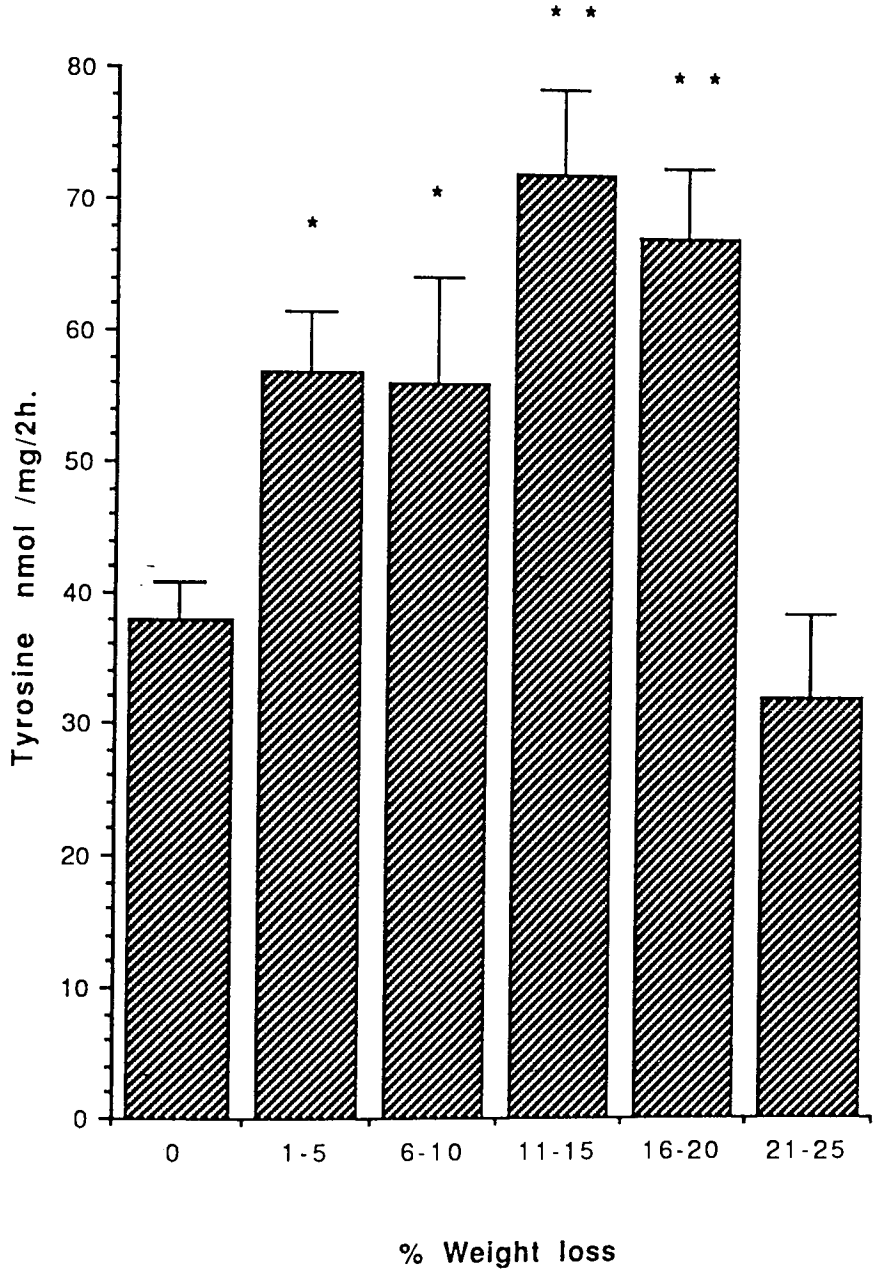
enzymes. Ottoson and Sylven (1960) reported changes in peptidases and proteolytic activity in the plasma of mice bearing ascites tumours - suggesting the presence of circulatory proteolytic factors. Similarly Beck and Tisdale (1987) have identified elevated levels of a serine protease in animals bearing the MAC16 tumour that may be responsible for enhanced muscle proteolysis. There are also a group of "factors" that mediate their effect by a more indirect mechanism - for example by enzyme induction. Enzyme activities in muscle tissue of tumour-bearing animals and humans show a depression of anabolic enzymes and an increased activity of catabolic enzymes such as the lysosomal enzyme cathepsin D. This increased activity of cathepsin D was demonstrated by Lundholm et al (1978) and correlated significantly with the fractional degradation rate of muscle proteins. The correlation between this enzyme activity and cancer was supported by the results of repeated measurements during the progression of the disease and after curative surgical procedures, after which the enzyme activity was normalised (Wesdorp, 1986).

The aim of this section was to establish whether the MAC16 adenocarcinoma produced a circulatory factor responsible for the increased muscle proteolysis associated with cachexia.

4.1.2. Results.

Figure 17.

The effect of serum from MAC16 tumour-bearing animals with progressive weight loss on tyrosine release from isolated gastrocnemius muscle.



Each bar represents the mean ± SEM of 4 animals. Differences were determined by one-way ANOVAR as *=P<0.05, **=P<0.01 from non tumour-bearing animals (group 0).

The results presented in fig.17 show the increasing levels of tyrosine release elicited from isolated gastrocnemius muscle preparations under the influence of serum from MAC16 tumour-bearing animals with progressive weight loss. The level of protein degradation increases up to a maximum at 11-15% weight loss and then decreases to a level comparable to that found in non tumour-bearing animals.

4.1.3. Discussion.

Beck and Tisdale (1987) have previously associated the increased loss of skeletal muscle in animals bearing the MAC16 tumour with increased plasma levels of a proteolysis-inducing factor - Belizario et al (1991) has also detected similar material in the plasma of cancer patients with in excess of 10% weight loss. The level of protolysis-inducing factor in cachectic MAC16 tumour-bearing animals increases progressively up to 20% weight loss and thereafter decreases. This suggests that the proteolytic factor may be responsible for the initiation of protein degradation, perhaps through a cascade of events that once started does not require further stimuli to proceed - thus at high weight losses the factor may not be required. The nature of the factor that increased protein degradation was further investigated in section 4.2., 4.3. and 4.6. For further discussion see appendix.

4.2. Identification of the relationship between muscle degradation in cancer cachexia and serum and tumour proteolytic enzymes.

4.2.1. General Introduction.

The muscle wasting associated with cancer cachexia can be extremely devastating - a patient can lose 30% of the muscle mass in three weeks and this will lead to rapid death through a depletion of cardiac and respiratory muscles (Shamberger, 1984, Lindsey, 1986). Theologides in 1976 proposed that the derangement of host metabolism was a direct consequence of the production of peptides, oligonucleotides or other metabolites by the tumour. This proposal was further substantiated by Norton et al in 1985 who demonstrated the parabiotic transmittal of humoural anorectic/cachectic factors in a rat tumour model, in which there was no evidence of metastases or endocrine function. Beck and Tisdale in 1987 demonstrated the existence of distinguishable lipolytic and proteolytic factors elaborated by the MAC16 adenocarcinoma.

In normal tissue proteolytic enzymes and their inhibitors play a crucial role in controlling a number of diverse physiological processes for example blood clotting, clot lysis, tissue remodelling, hormone processing, ovulation, embryo implantation and in the induction of selective DNA

amplification (Roblin, 1978, Hart and Rehemtulla, 1988, Stubblefield and Brown, 1976). Malignant tissue was first shown to possess abnormal proteolytic activity by Fischer in 1925. He demonstrated the ability of malignant cells to lyse plasma clots while normal tissue did not. The malignant state of a tumour is defined as the ability of the tumour cells to detach from the primary growth, invade surrounding tissues, and to spread to distant sites giving rise to secondary foci of growth (metastases). This can lead to massive tissue destruction and death (Recklies et al, 1980). Proteolytic enzymes have been implicated in the key roles of metastasis, in angiogenesis and also in the loss of growth control of tumour cells (Quigley, 1979). The ability of some tumours and tumour cell-lines to actively secrete collagenolytic enzymes has been shown (Dresden et al, 1972, Dabbous et al, 1977, Kuettner et al, 1977). There has also been much interest in the induction and secretion of cathepsin D from oestrogen receptor positive cell lines and tumours. This protease can degrade extracellular matrices and proteoglycans, promote growth in vitro and high levels may be correlated with poor prognosis in breast cancer (Thorpe et al, 1989, Scambia et al, 1991). The plasminogen activators have been implicated in many aspects of cancer - Wang et al (1980), and Reich et al (1988) have shown that plasminogen activator activity correlates with metastatic potential. Unkeless et al (1974) have demonstrated that the fibrinolytic system is

consistently associated with oncogenic transformation in cell cultures. The increase in plasminogen activation associated with this transformation determines part of the phenotypic properties of the transformed cells including colony formation in a semi-solid media and the characteristic changes in cell morphology and migration.

It has been shown (Schelp and Pongpaew, 1988) that populations with a diet low in calories, fat and animal protein, but high in vegetables and fibre, have a low incidence of cancer of the colon, rectum, breast and prostate. It is proposed that such a diet protects against cancer by stimulating an increase in endogenous proteinase inhibitors. Alternatively Troll et al (1987) and Yavelow et al (1983) suggest that similar epidemiological studies are attributable to the ingestion of chemopreventative agents occuring naturally in vegetables. Protease inhibitors occur in plants as a defense mechanism against marauding insects - they act by inhibiting the insects digestive enzymes, thus preventing damage to the stem and leaves. It is thought that the production of protease inhibitors by the host may help to counteract the overproduction of amino acids elicited from the host by the tumour. (The role of acute phase proteins is discussed in section 4.6.). Protease inhibitors can interfere with cancer development in a number of ways - they can inhibit carcinogen-induced chromosomal aberations (Kinsella and

Radman, 1980), inhibit transformation of fibroblasts after transfection with an activated H-ras oncogene (Garte et al, 1987) and inhibit Ehrlich ascites tumour growth (Verloes et al, 1978). In addition some of the metastatic processes can be inhibited - for example rabbit antibodies against human urinary urokinase inhibited the metastasis of human carcinoma HEp3 cells across the chorioallantoic membrane to the chick embryo (Ossowski and Reich, 1983). Cartilage and aorta derived proteins of low molecular weight have been shown to inhibit collagenase, cathepsin B, papain and trypsin thereby inhibiting metastasis and neovascularisation (Brem et al, 1976, Rifkin and Crowe, 1977, Roughley et al, 1978) and the peritumoural administration of cysteine, an inhibitor of collagenase, caused an increase in the survival time of mice bearing a malignant thymoma (Campbell et al, 1974).

Guanidinobenzoatase was first described by Steven et al (1981) as a cell surface protease with the ability to cleave α-N-benzoyl D-L-arginine-β-naphthylamide N-carbobenzoxy-L-tyrosine nitrophenyl ester, N-benzoyl-arginine ethyl ester and casein. The trypsin-like, arginyl specific enzyme guanidinobenzoatase was so called because of its ability to cleave the guanidinobenzoates, p-nitrophenyl-p-guanidinobenzoate and 4-methylumbelliferyl-p-guanidinobenzoate at the common moiety i.e. guanidinobenzoate to yield the fluorescent

products p-nitrophenol and 4-methyl umbelliferone respectively (Steven and Al-Ahmad, 1983). These substances were designed specifically as active-site titrants to quantify and inhibit trypsin, but they are in fact suicide substrates for enzymes such as trypsin, thrombin and plasmin (Steven et al, 1988a).

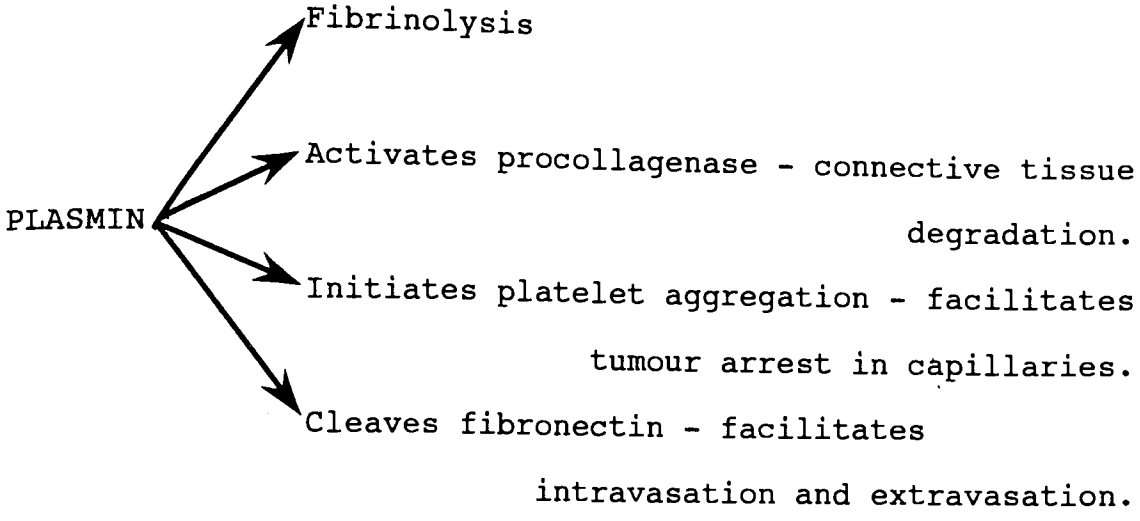
Guanidinobenzoatase has been shown to play a role in the activation of the zymogen of collagenase and is capable of degrading fibronectin - a major glycoprotein component of the extracellular matrix. Purified guanidinobenzoatase cleaves the peptide GLY-ARG-GLY-ASP which is known to be concerned with the attachment of cells to fibronectin - consequently guanidinobenzoatase has been implicated in metastasis (Steven et al, 1988b). Guanidinobenzoatase was originally thought to be specifically confined to tumour cells, but as more studies were carried out with the fluorescent probes 9-aminoacridine and Nα-dansyl-homoarginine it was found that it had a more generalised distribution, i.e. in infiltrating lymphocytes, squamous epithelial cells, hair follicles, cells of newly proliferating blood vessels, fetal cells, cartilage, normal mouse serum, spermatozoa and seminal plasma (Steven et al, 1985, 1986). The fluorescent probes showed a marked staining differential in tissues, the leading edges of highly invasive tumours e.g. metastatic melanomas stained very intensely whereas benign naevus cells stained only

weakly (Steven et al, 1987). The fluorescent probes bound to guanidinobenzoatase by competitive inhibition and the differences in staining have been demonstrated to be caused by the presence or absence of an inhibitor. It is thought that the control of cell surface guanidinobenzoatase by inhibitors may be one of the regulatory mechanisms by which benign cells are prevented from developing into malignant cells or developing metastatic capabilities. A number of inhibitors of guanidinobenzoatase have been identified - for example low molecular weight inhibitors of trypsin, fresh extract of colon or lung tissue, concentrated human urine, the fluorescent probes 9-aminoacridine and Nα-dansyl-homoarginine and BZAR [bis-(N-benzyloxycarbonyl-L-arginamido)-rhodamine]. The in vitro inhibitors of guanidinobenzoatase can be modified by oxidation by air or by oxidised glutathione or potassium permanganate resulting in a conformational change, which enables the fluorescent probe to be bound, i.e. converting the enzyme from a latent to an active form (Steven and Griffin, 1988c, Steven et al, 1988d). The important implications of this are that if the extracellular oxidising potential is increased the tumour cells metastatic potential may also increase. This may relate to the fact that metastasis is usually associated with well vascularised tumours. The competitive inhibition of guanidinobenzoatase by the fluorescent probes and inhibitors could perhaps be exploited to target cytotoxic

drugs to the tumour. Steven et al in 1989 demonstrated in vitro the delivery of an adriamycin-agmatine complex to the active centre of guanidinobenzoatase associated with invasive tumour cells located in the lymph nodes and in squamous cell carcinoma of the oral cavity.

Steven and Hill in 1988e showed that exposure to the fibrous mineral erionite, a carcinogen known to cause mesothelioma, induced the expression of guanidinobenzoatase in mesothelial cells of rats initially lacking the enzyme. Within hours the number of cells possessing the enzyme steadily increased until the animals died with massive pleural tumours. It has also been shown that the transplantation of human colonic tumour cells, with no guanidinobenzoatase activity, into nude mice caused an induction of guanidinobenzoatase in the tumour (Steven et al, 1990).

There is speculation that guanidinobenzoatase is tissue-type plasminogen activator (tPA). Guanidinobenzoatase and tPA share a number of homologies - tPA together with its inhibitors play a central role in many biological processes - particularly in extracellular proteolysis (Roblin, 1978). TPA is an arginine specific protease that converts the serum pro-enzyme plasminogen into the broad specificity protease plasmin (Quigley et al, 1974).



Plasmin can hydrolyse a variety of proteins from fibrin and fibronectin, to immunoglobulin and complement components. Once generated plasmin activity is eventually inhibited by serum plasmin inhibitors such as α_1 antitrypsin, α_2 macroglobulin, C1-inactivator, antithrombin 3 and α_2 antiplasmin. Thus the fibrinolytic capacity of serum is determined by the balance between profibrinolytic and antifibrinolytic activity. Two major forms of plasminogen activator exist - urokinase-like (u-PA) and tPA. They are the products of different genes and differ biochemically and immunochemically. The function of u-PA is more related to cellular function and tissue remodelling, whereas tPA is a major regulator of fibrinolysis. u-PA is found in large quantities in the urine whilst tPA has a wider distribution - it is found in primary cultures of spontaneous, virally and chemically induced animal neoplasms and in several human tumour cell lines (Nagy et al, 1977). It is also found in or released from the kidney, granulocytes,

activated macrophages, sperm, seminal plasma, endometrial fluid and in numerous cell lines of non-malignant origin (Hart and Rehemtulla, 1988, Rondeau et al, 1986). In addition to fibrinolysis and cellular migration tPA and u-PA also participate in hormone processing, ovulation and embryo implantation.

A number of studies have recently shown that cell surface guanidinobenzoatase is very similar to single chain tissue-type plasminogen activator in two major ways - polyclonal antibodies recognising tPA inhibit 9-amino acridine binding to guanidinobenzoatase and specific protein inhibitors of tPA similarly block binding (Steven et al, 1990, 1991). However, Perry and Scott (1990) have demonstrated that the immunochemical inhibition of plasminogen activator did not affect the hydrolysis of p-nitrophenyl guanidinobenzoate by guanidinobenzoatase.

Steven and Al-Ahmad (1983) originally referred to the tumour cell surface enzyme as guanidinobenzoatase. However, a soluble form of the enzyme was also found in the ascitic fluid of Ehrlich ascites tumours grown in mice. Subsequently an enzyme was identified in normal mouse serum that behaved similarly to the guanidinobenzoatase in ascitic fluid in its ability to cleave 4-methylumbelliferyl-p-guanidinobenzoate and 4-nitrophenyl-p-guanidinobenzoate. The mouse serum enzyme

shows some similarity to a proteolytic factor associated with a cachexia-inducing murine tumour (MAC16)(Beck and Tisdale, 1987) in being a serine protease present in serum and not being inhibited by the trypsin inhibitor.

The aim of this section is to attempt to determine the role, if any, of guanidinobenzoatase in the cachexia of cancer, by determining the relationship of this enzyme to the development of the condition in the MAC16 murine model. The polyunsaturated fatty acid (PUFA) eicosapentaenoic acid, which has a pronounced anticachectic/antitumour effect in animals bearing the MAC16 tumour (Tisdale and Beck, 1991) and has also been shown to reduce the invasive and metastatic activities of malignant tumour cells (Reich et al, 1989) has been assessed and compared with bis(carbobenzyloxy carbonyl-L-arginamido)-rhodamine (BZAR), a known inhibitor of the enzyme (Steven et al, 1988a) and other PUFAs, on their effect on the activity of guanidinobenzoatase.

4.3. Identification of the relationship between guanidinobenzoatase and cancer cachexia.

4.3.1. Results.

4-methyl-umbelliferyl-p-guanidinobenzoate was cleaved by serum from non tumour-bearing NMRI mice to yield the

Figure 18A.

Extent of release of methylumbelliferone (MU) from 4-methylumbelliferyl-p-guanidinobenzoate (MUGB) by serum from non tumour-bearing, NMRI animals.

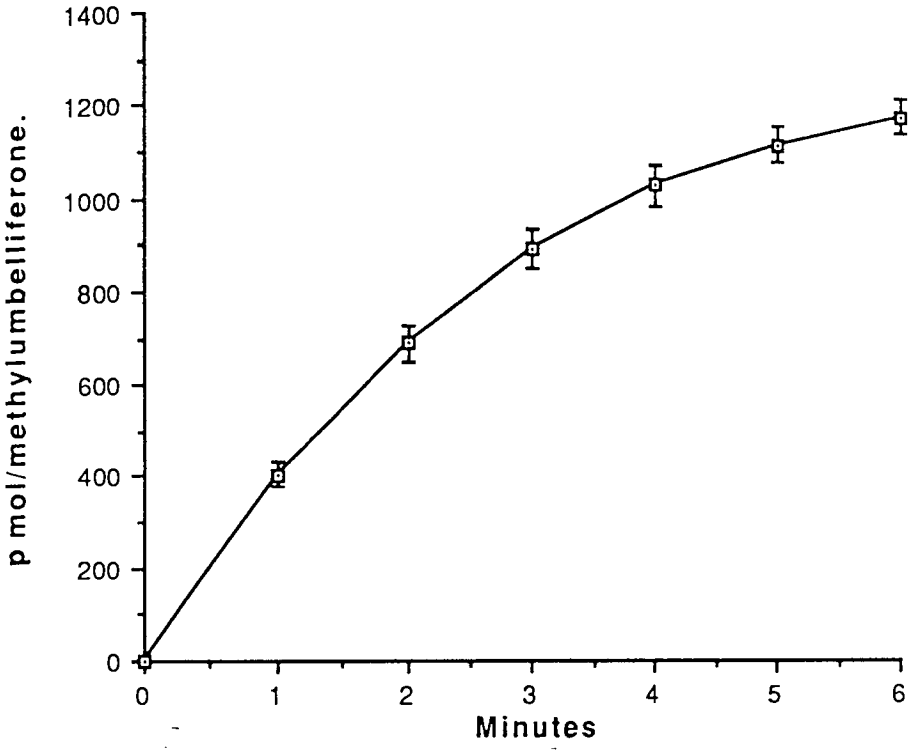
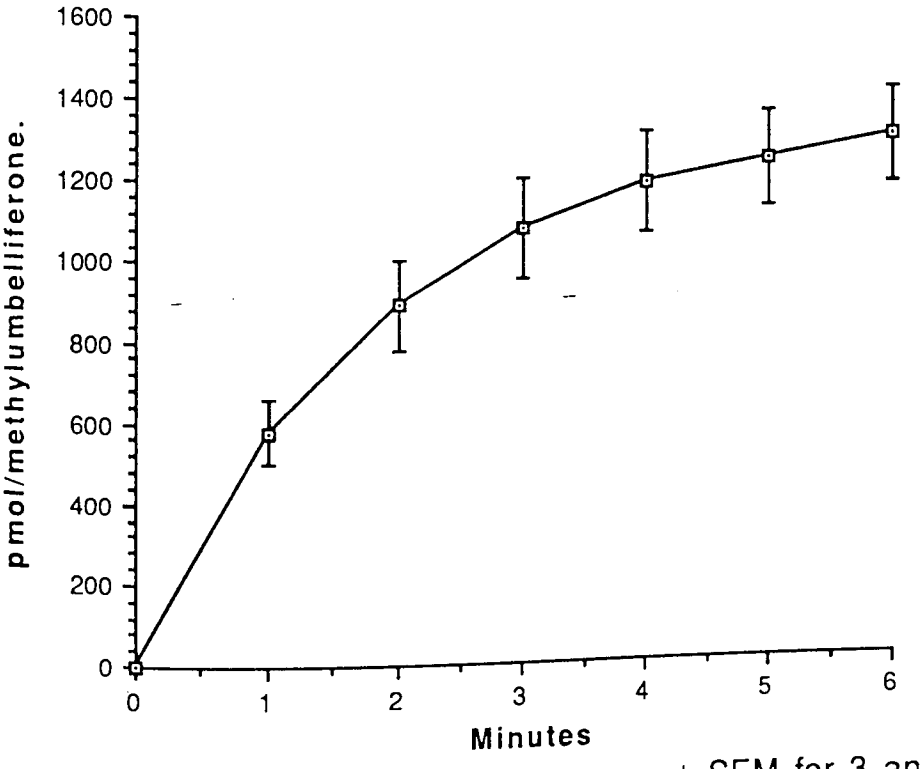


Figure 18B.

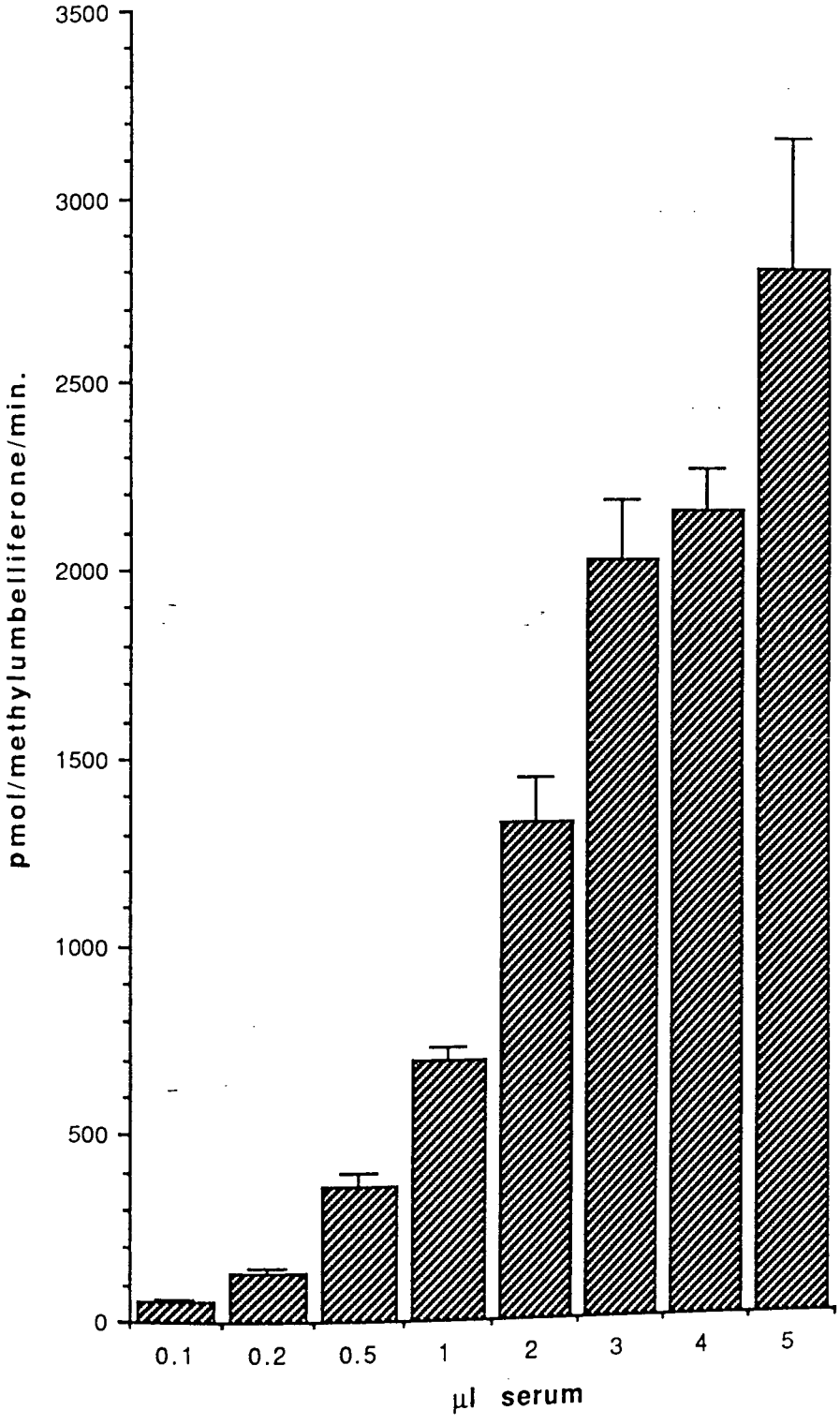
Extent of release of MU from MUGB by serum from MAC16 tumour-bearing animals without weight loss.



Results are expressed as the mean ± SEM for 3 animals.

Figure 19.

Extent of release of MU from MUGB by aliquots of serum from non tumour-bearing, NMRI animals.



Each bar represents the mean ± SEM of 3 experiments.

fluorescent product 4-methylumbelliferone (Fig. 18A). The intensity of the fluorescence increased with progressive incubation time with a linear time course over the first 3-4 min and was proportional to the volume of serum in the assay over the range of 0 to 5µl (Fig. 19). The serum and tumour enzyme level was similar in non-cachectic and cachectic MAC16 and MAC13 tumour bearing animals (Fig. 18B, 20A and 20B), (Fig. 21A, 21B and 23A) - however the tumour enzyme level was about 100-fold less than the comparable serum level. The serum and tumour enzyme levels were not affected by progressive weight loss (Fig. 22A, 22B). Human serum also contained guanidinobenzoatase, though at a level about 50-fold lower than in mouse serum. Again there was no difference between non-cachectic (3 patients with breast cancer, high grade lymphoma and Hodgkins disease) and cachectic (3 patients with breast cancer, non-Hodgkins lymphoma and malignant teratoma, weight loss 14, 7 and 5kg respectively) patients or between cancer patients and control subjects. (cachectic 15.6 $\pm$ 4.3; non-cachectic 15.6 $\pm$ 3.2; control 20.3 $\pm$ 3.3 pmol methylumbelliferone/2min/5µl serum) (Fig. 23B, 24A and 24B). The DEAE cellulose fractionation of control mouse serum under the influence of a salt gradient (Fig.25) illustrates that the enzyme cleaving 4-methylumbelliferyl-p-guanidinobenzoate occurs in a fairly discrete fraction - eluting between 0.1M and 1.8M NaCl. However, the pattern of activity seen with fractions of

Figure 20A.

Extent of release of MU from MUGB by serum from MAC16 tumour-bearing animals with weight loss.

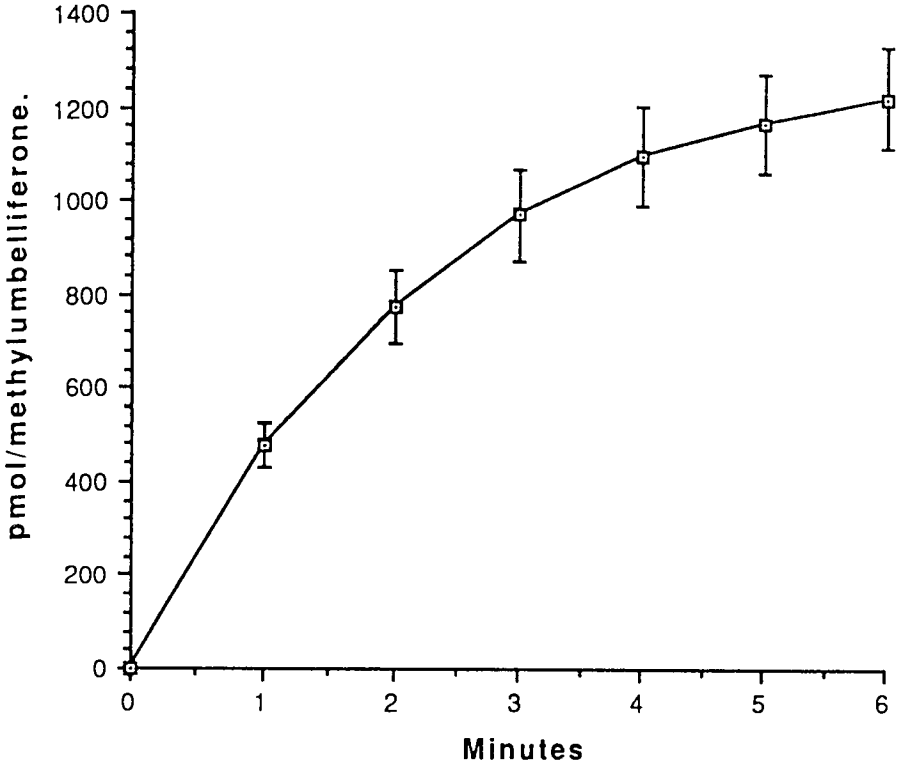
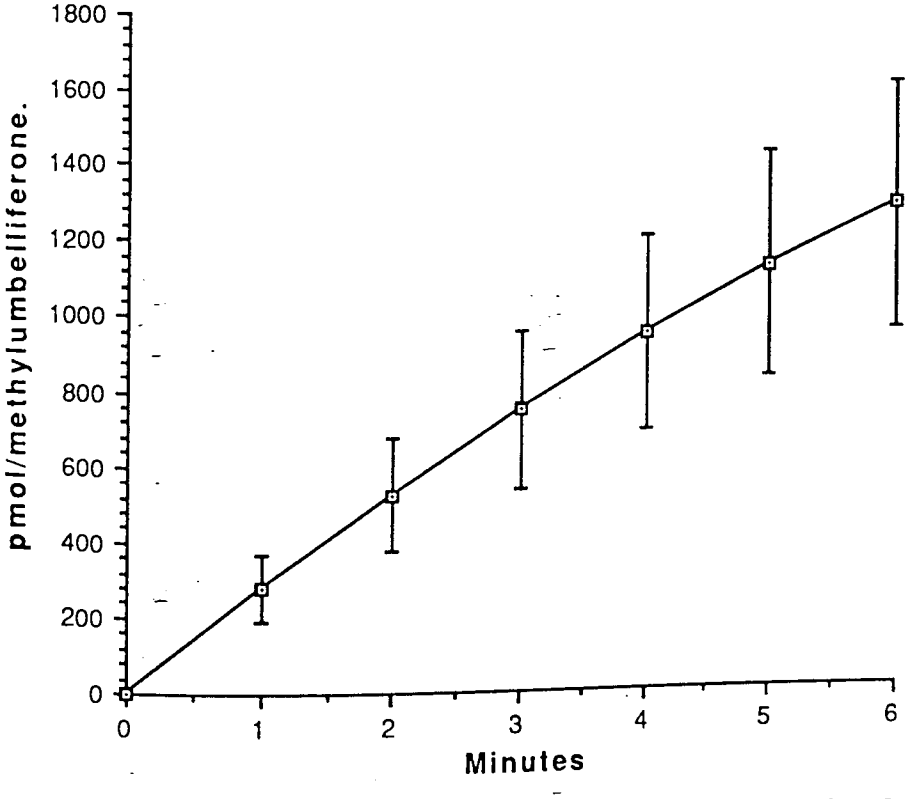


Figure 20B.

Extent of release of MU from MUGB by serum from MAC13 tumour–bearing animals.



Results are expressed as the mean ± SEM for 3 animals.

Figure 21A.

Extent of release of MU from MUGB by homogenates of MAC16 tumours from animals without weight loss.

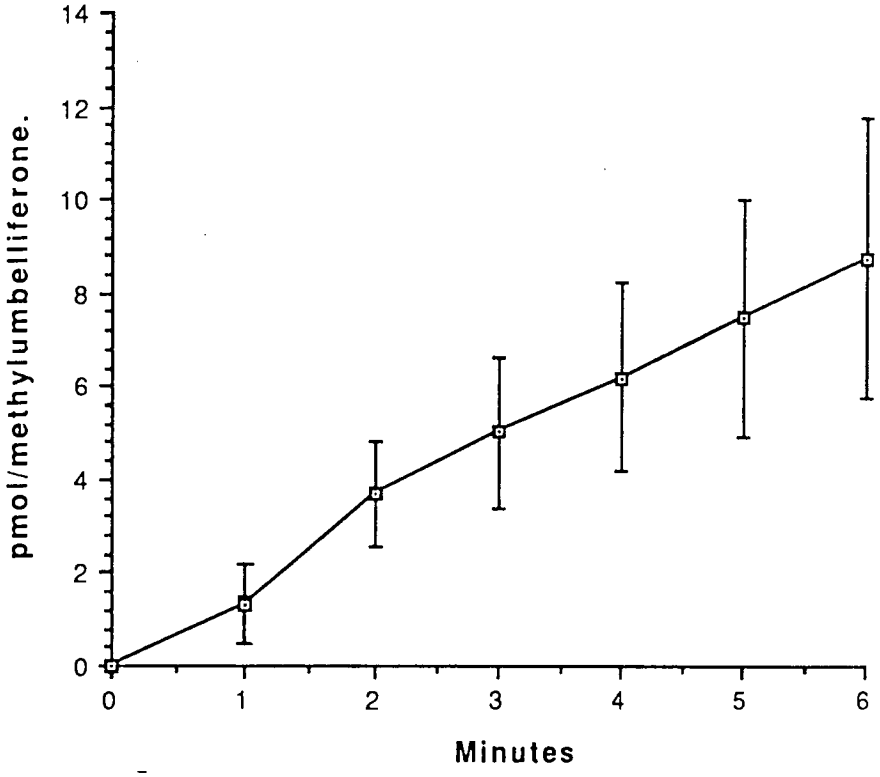
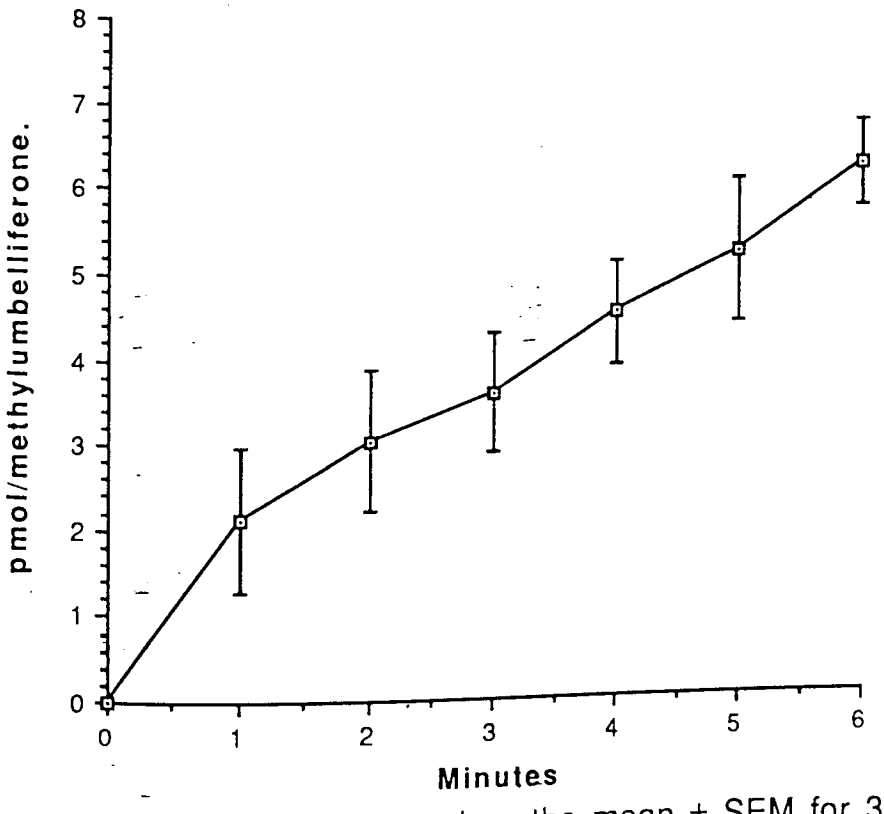


Figure 21B.

Extent of release of MU from MUGB by homogenates of MAC16 tumours from animals with weight loss.



Results are expressed as the mean ± SEM for 3 animals.

Figure 22A.

Extent of release of MU from MUGB by serum from MAC16 tumour-bearing animals with progressive weight loss.

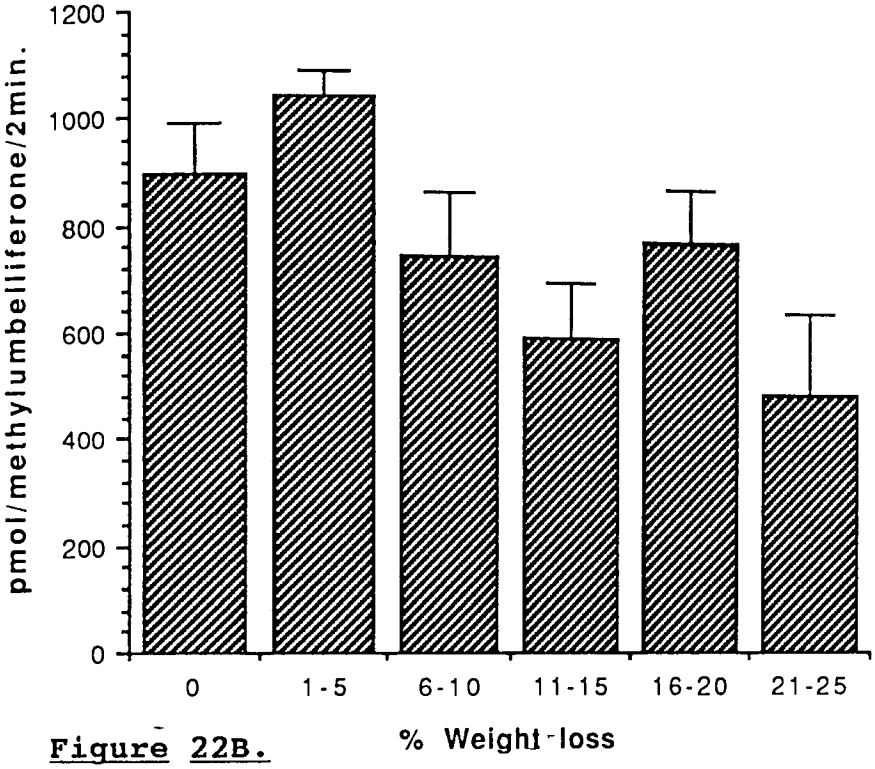
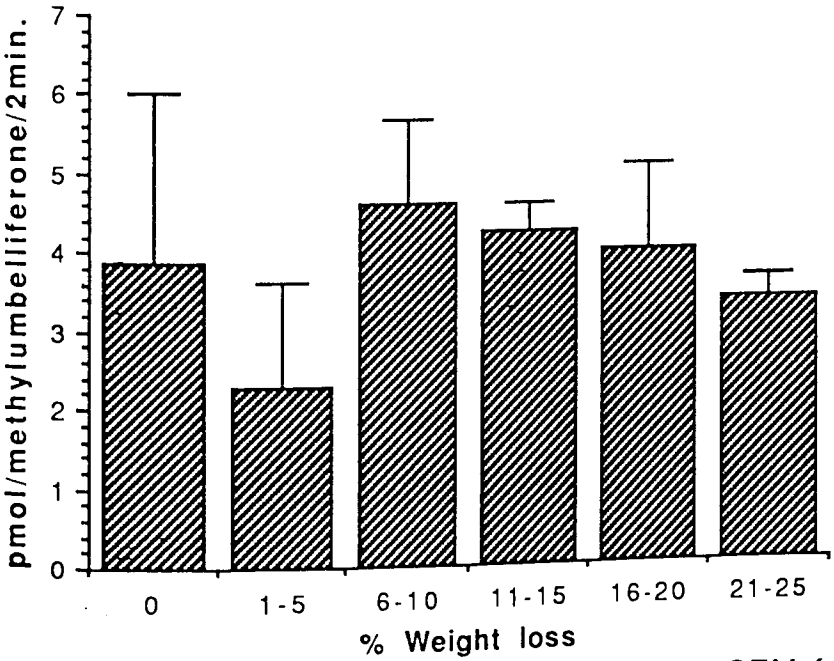


Figure 22B.

The extent of release of MU from MUGB by homogenates of MAC16 tumours from animals with progressive weight loss.



Results are expressed as the mean ± SEM for 4 animals. There are no significant differences between groups using one-way ANOVAR.

Figure 23A.

Extent of release of MU from MUGB by homogenates of the MAC13 tumour.

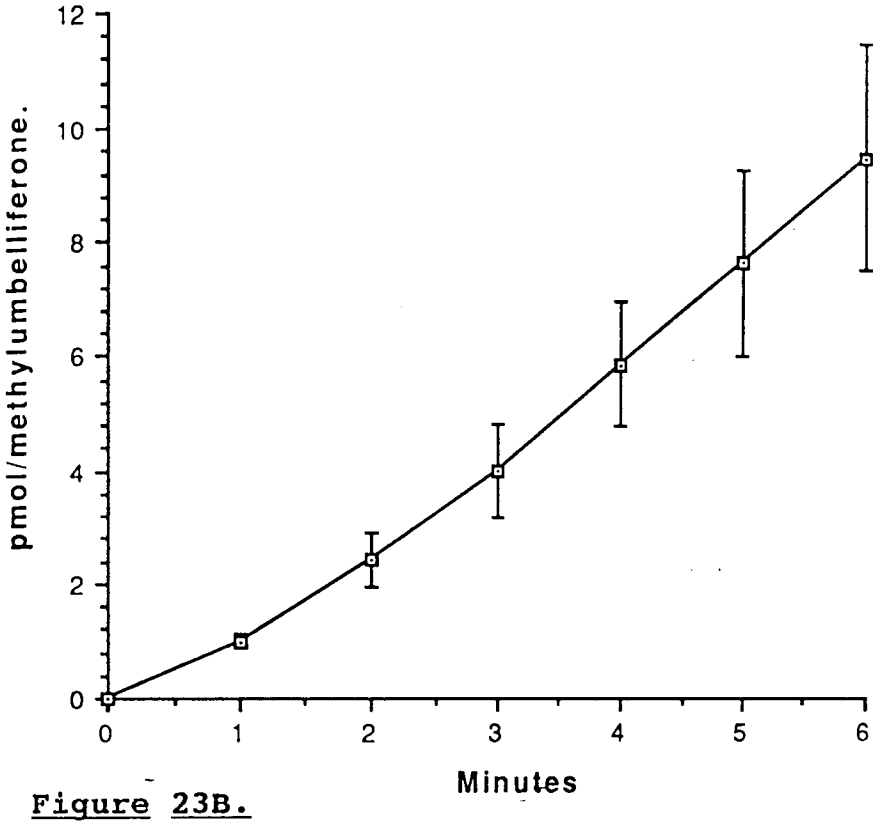
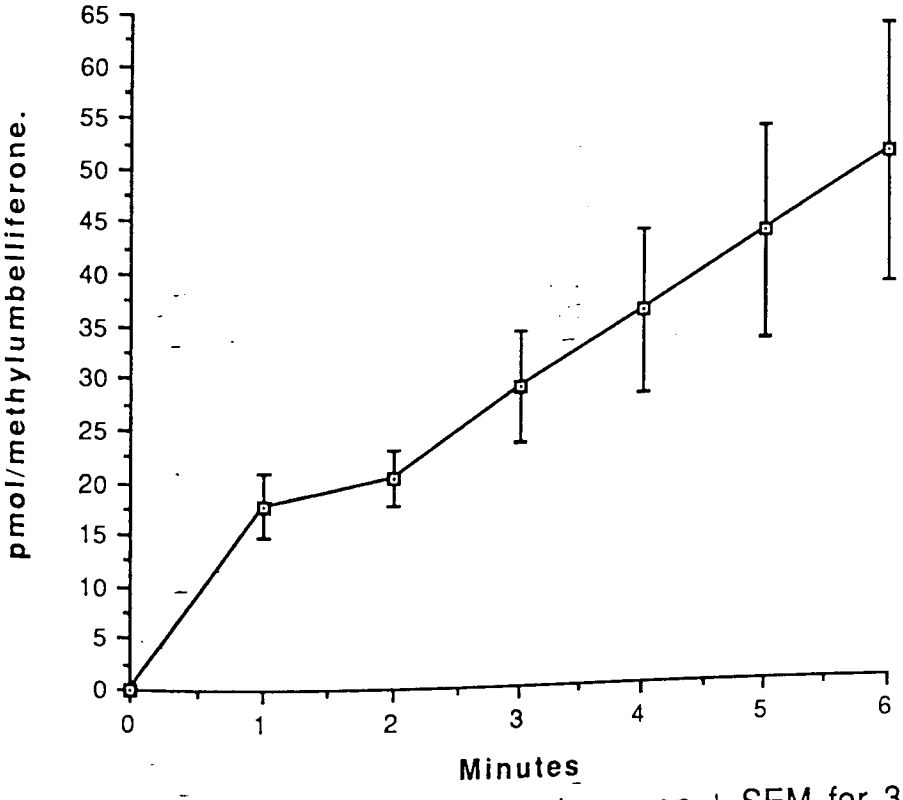


Figure 23B.

Extent of release of MU from MUGB by serum from control humans.



Results are expressed as the mean ± SEM for 3 experiments.

Figure 24A.

Extent of release of MU from MUGB by serum from cancer patients without weight loss.

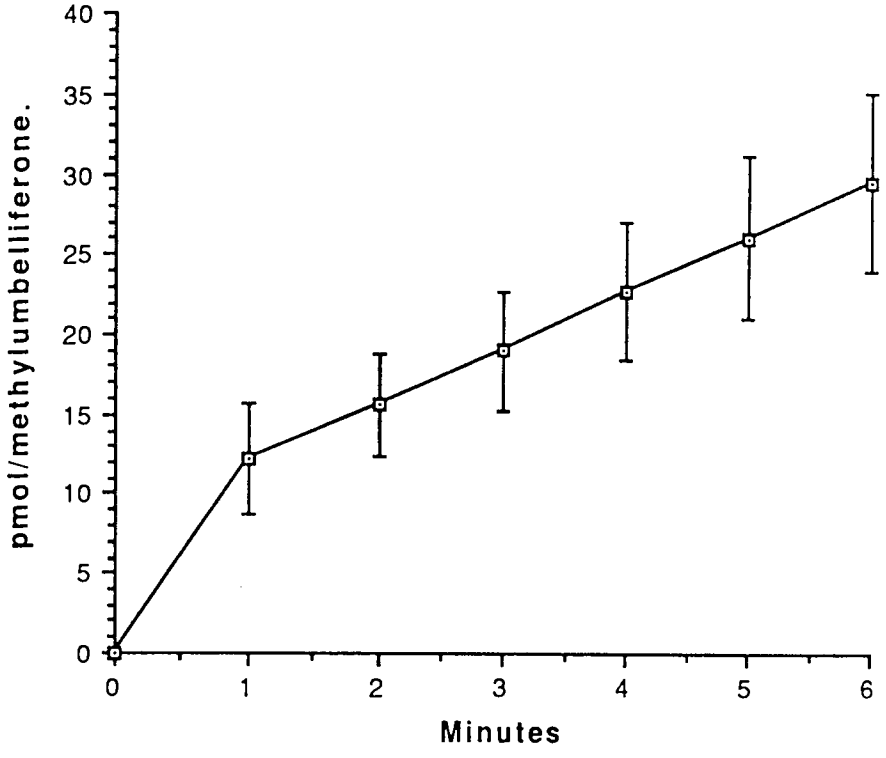
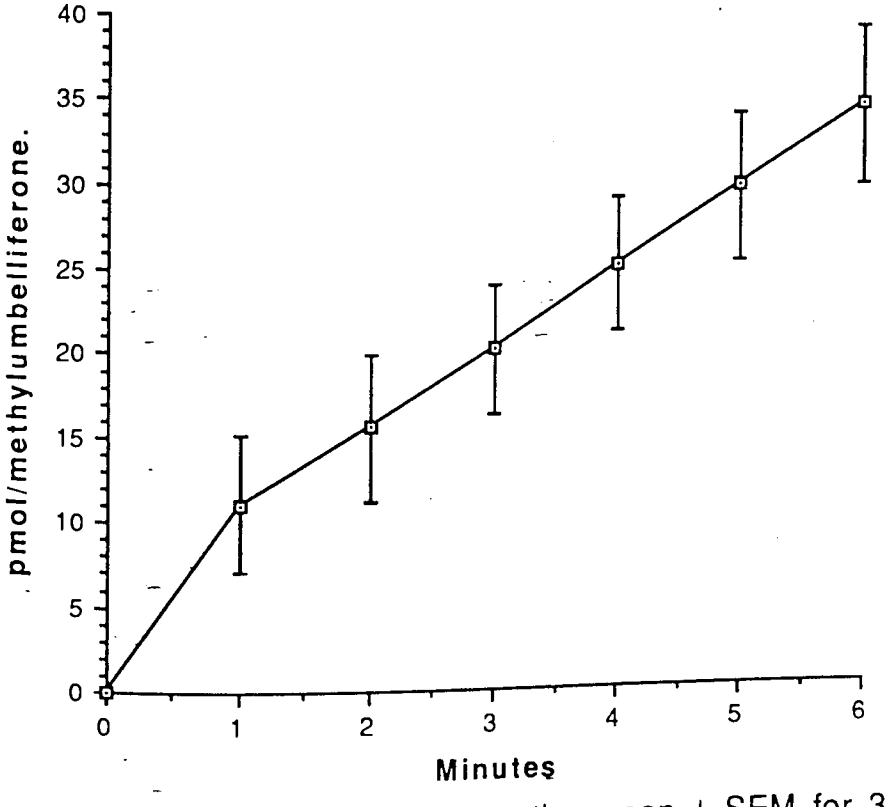


Figure 24B.

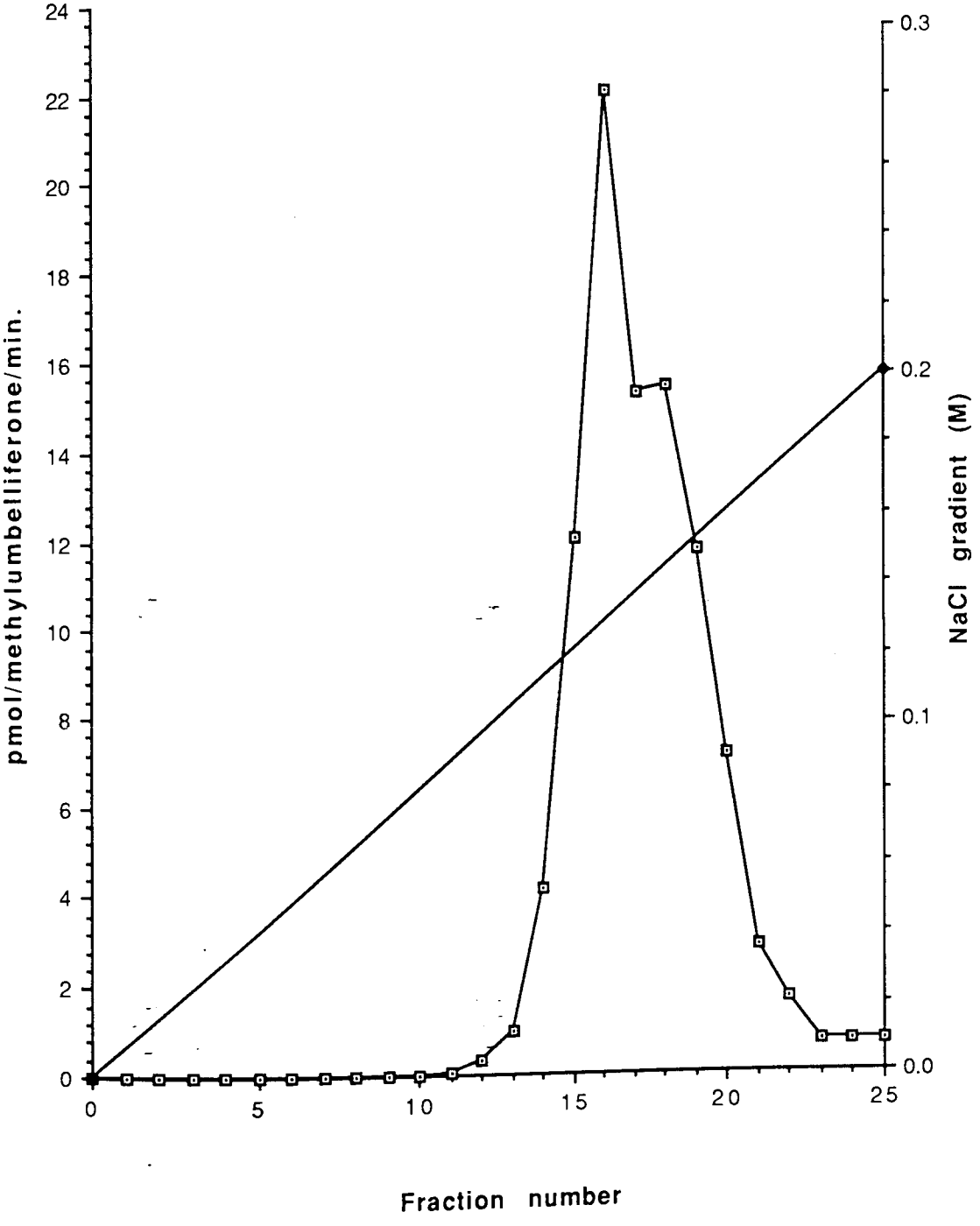
Extent of release of MU from MUGB by serum from cancer patients with weight loss.



Results are expressed as the mean ± SEM for 3 patients.

Figure 25.

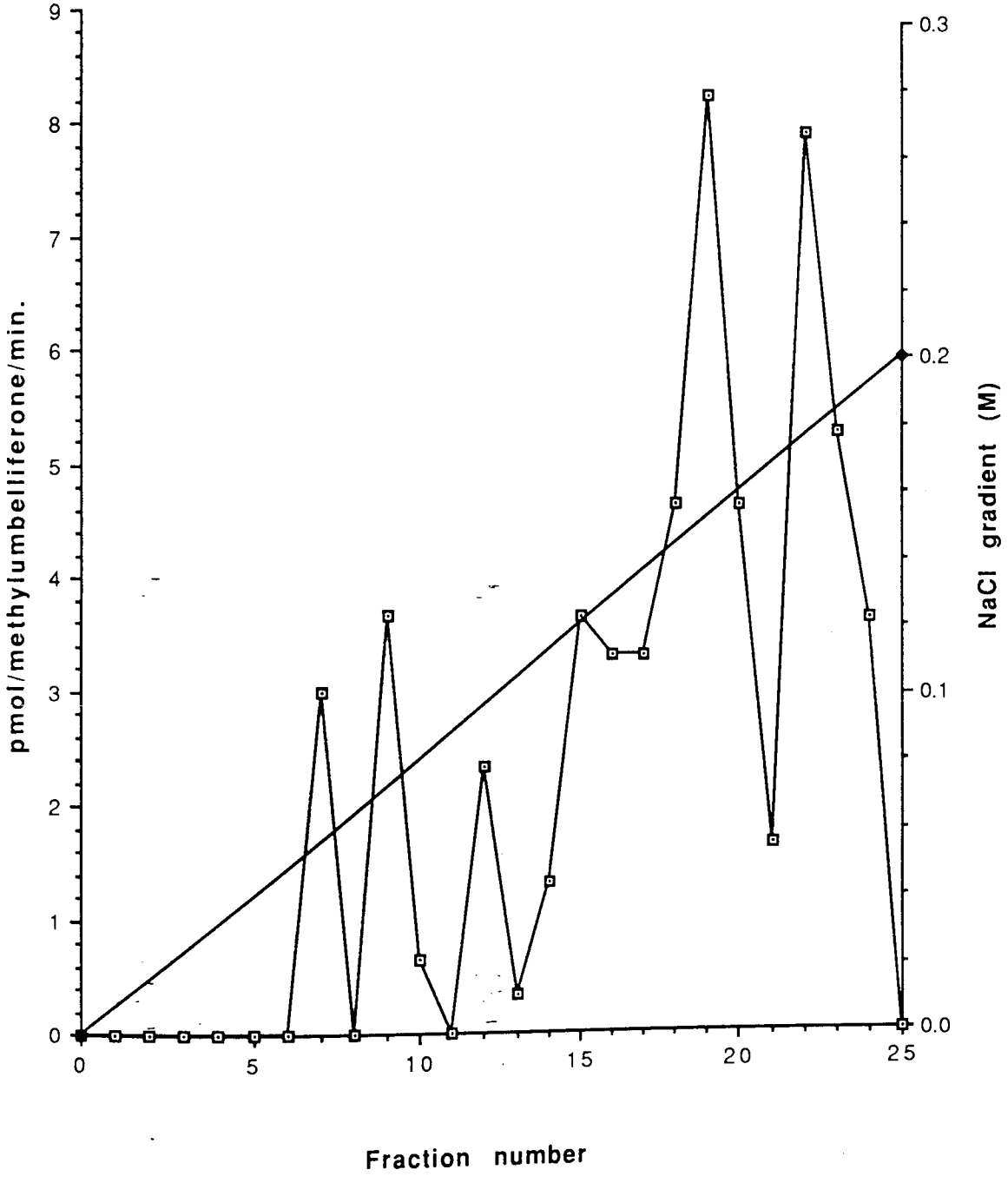
Fractionation of NTB mouse serum on a DEAE cellulose column.



Distribution pattern of MU released from MUGB by 1ml fractions eluted under a salt gradient with 0.1M phosphate buffer pH8.0. 1ml of NTB mouse serum was applied to the column.

Figure 26.

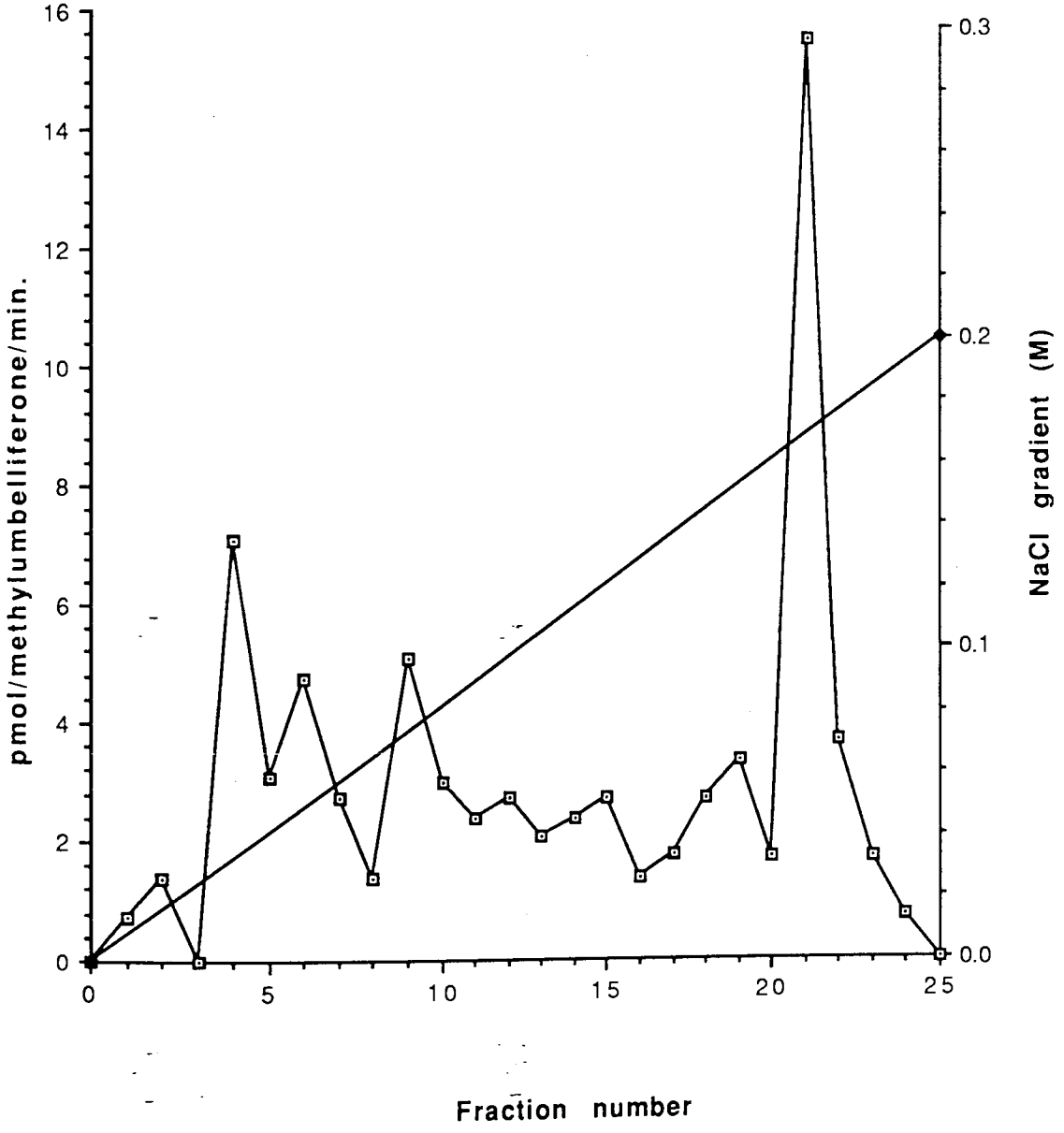
Fractionation of cancer patient serum with weight loss on a DEAE cellulose column.



Distribution pattern of MU released from MUGB by 1ml fractions eluted under a salt gradient with 0.1M phosphate buffer pH8.0. 1ml of NTB mouse serum was applied to the column.

Figure 27.

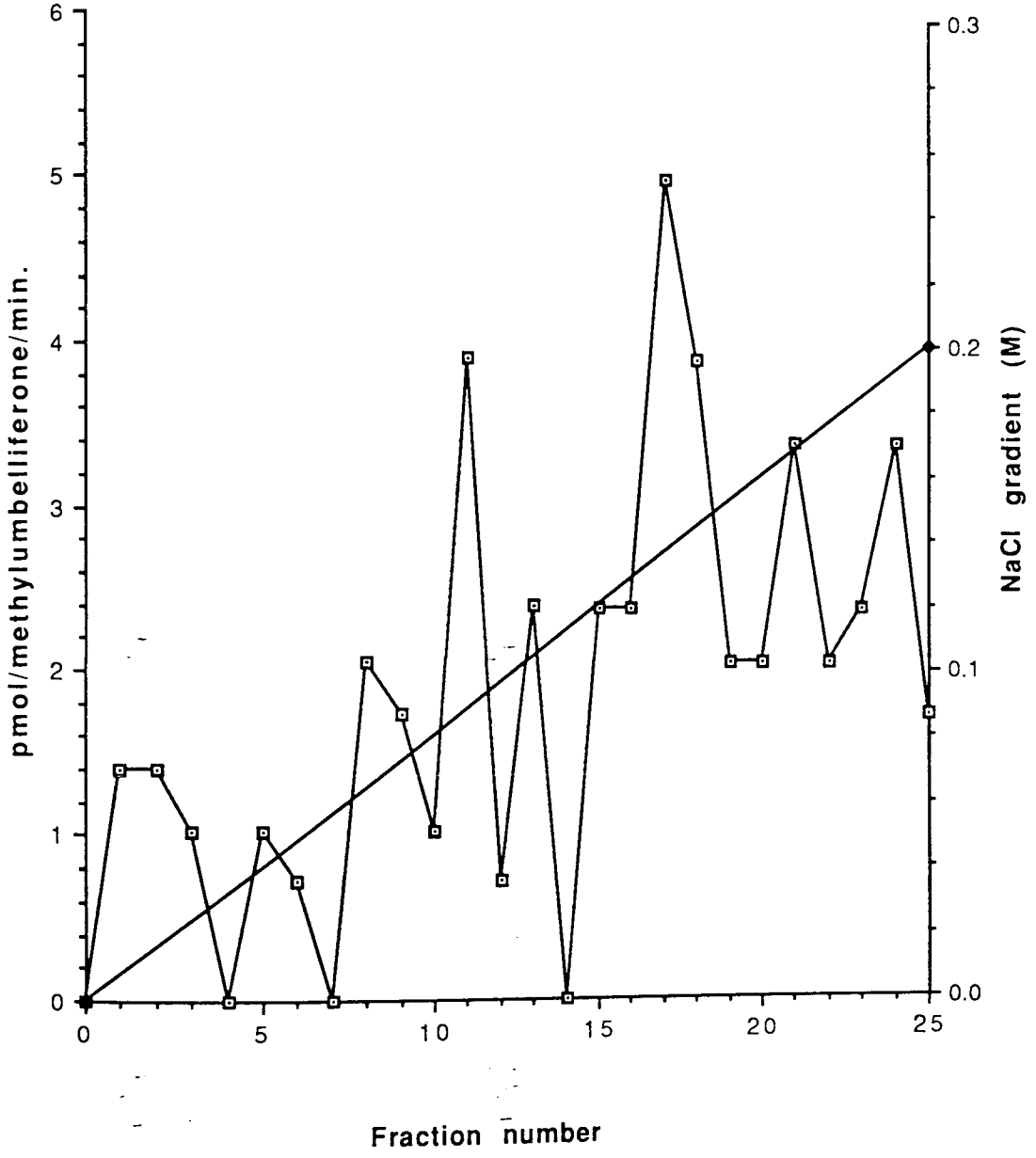
Fractionation of MAC16 tumour homogenate from animals with weight loss on a DEAE cellulose column.



Distribution pattern of MU released from MUGB by 1ml fractions eluted under a salt gradient with 0.1M phosphate buffer pH8.0. 1ml of MAC16 tumour homogenate was applied to the column.

Figure 28.

Fractionation of MAC 13 tumour homogenate on a DEAE cellulose column.



Distribution pattern of MU released from MUGB by 1ml fractions eluted under a salt gradient with 0.1M phosphate buffer pH8.0. 1ml of MAC13 tumour homogenate was applied to the column.

cachectic cancer patient serum (Fig.26), cachectic MAC16 tumour homogenate (Fig.27) and MAC13 tumour homogenate (Fig.28) was more dispersed but the majority of activity again eluted approximately between 0.1M and 1.8M NaCl.

4.3.2. Discussion.

These results show that there is no correlation with the tumour-bearing state or the appearance of cancer cachexia and the level of guanidinobenzoatase in the serum of mice or humans or in MAC16 and MAC13 tumour homogenates, as assayed using 4-methylumbelliferyl-p-guanidinobenzoate as substrate. This is despite the fact that the MAC16 tumour cells contain active guanidinobenzoatase on their surface as detected with 9-aminoacridine as previously observed with other tumour cells (Steven et al, 1987,1988). The tumour homogenates were probably not as active as had first been thought, because of the presence of inhibitors - this may also be the case in the human serum experiments. Human serum contains at least seven protein inhibitors of trypsin-like enzymes and in particular a fast-acting inhibitor of tissue-type plasminogen activator that is not identical to α2-antiplasmin or α2-macroglobulin (Verheijen et al, 1984) - these inhibitors form part of the acute phase proteins (described in section 4.6.). However, there is considerable species specificity; mouse serum protease

inhibitors may not be as effective at inhibiting guanidinobenzoatase as their human counterparts. The results from the fractionated sera and tumour homogenates suggest that there are a number of substances that react enzymically with the guanidinobenzoatase substrate to yield methylumbelliferone. The major cleaving component seen after ion exchange chromatography of control mouse serum is also present in cachectic cancer patient serum and also in the tumour homogenates.

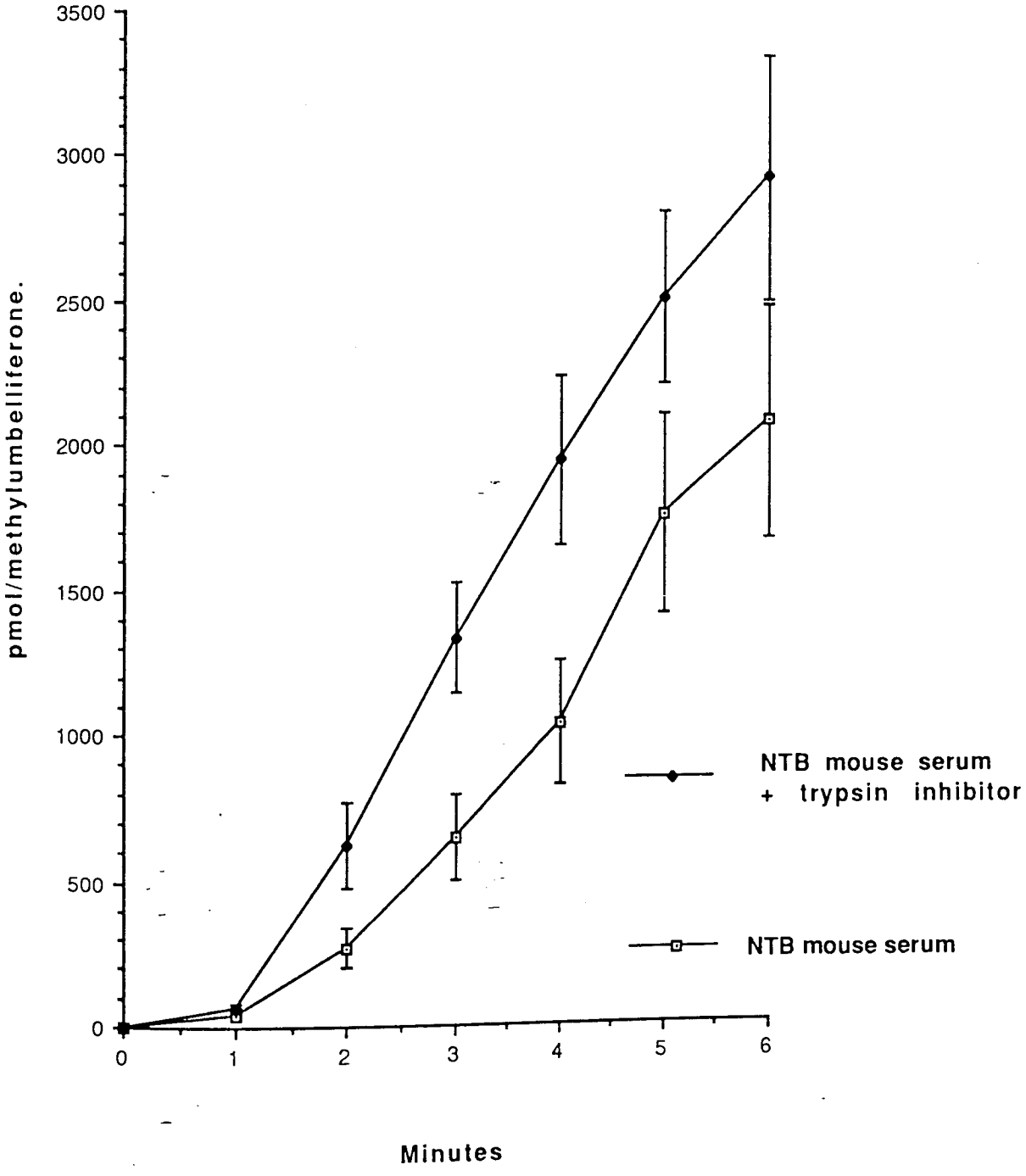
4.4. Identification and characterisation of the relationship between soluble and cell-bound guanidinobenzoatase, tissue-type plasminogen activator, esterase and trypsin - using inhibition studies.

4.4.1. Results.

It was considered that some guanidinobenzoatase activity could be attributed to trypsin or trypsin-like enzymes. However, the addition of trypsin inhibitor to control mouse serum as seen in Fig.29 seemed to stimulate the activity of the enzyme. The effect of polyunsaturated fatty acids on control mouse serum guanidinobenzoatase is shown in Fig. 30-33. All of the polyunsaturated fatty acids inhibited guanidinobenzoatase at μM concentrations which were in the same range as that found for bis(carbobenzyloxycarbonyl-L-arginamido)-rhodamine (BZAR)

Figure 29.

The enhancement of guanidinobenzoatase activity by trypsin inhibitor.



Results are expressed as the mean ± SEM for 3 animals.

Figure 30A.

The inhibition of guanidinobenzoatase by EPA dissolved in equimolar lysine.

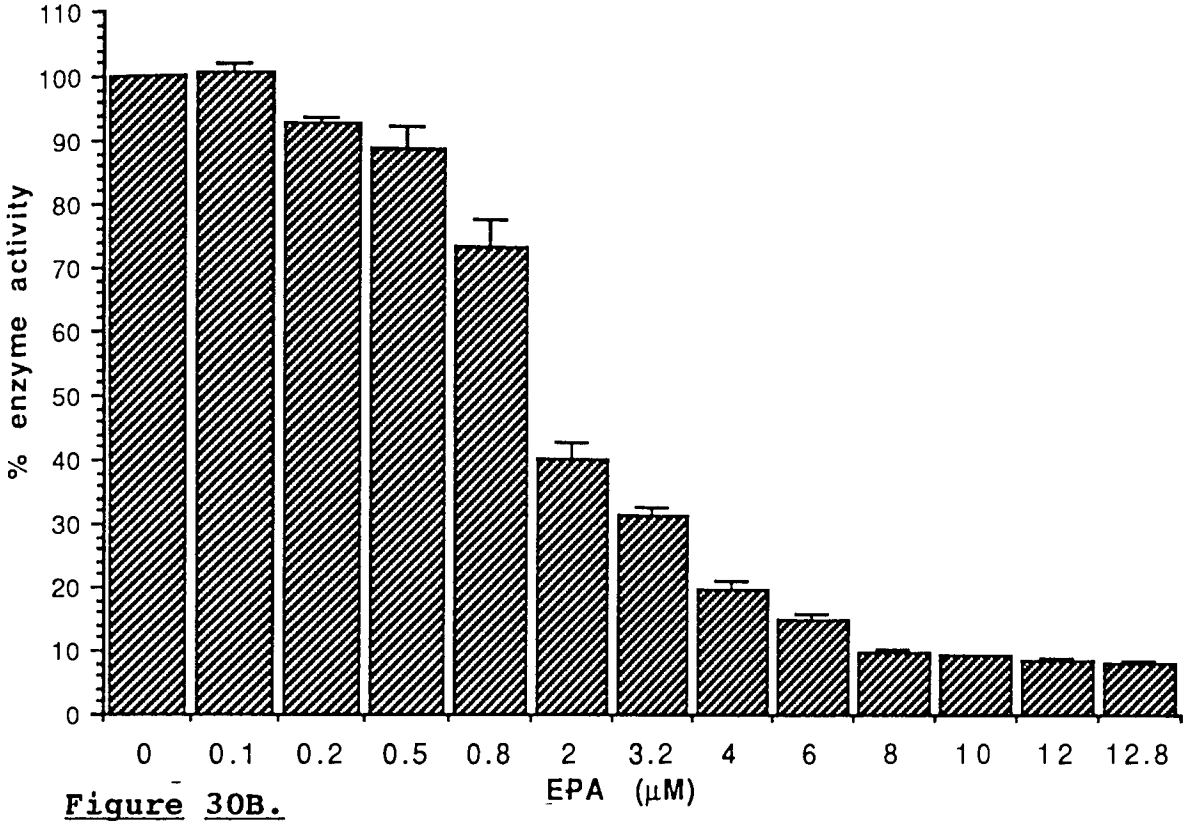
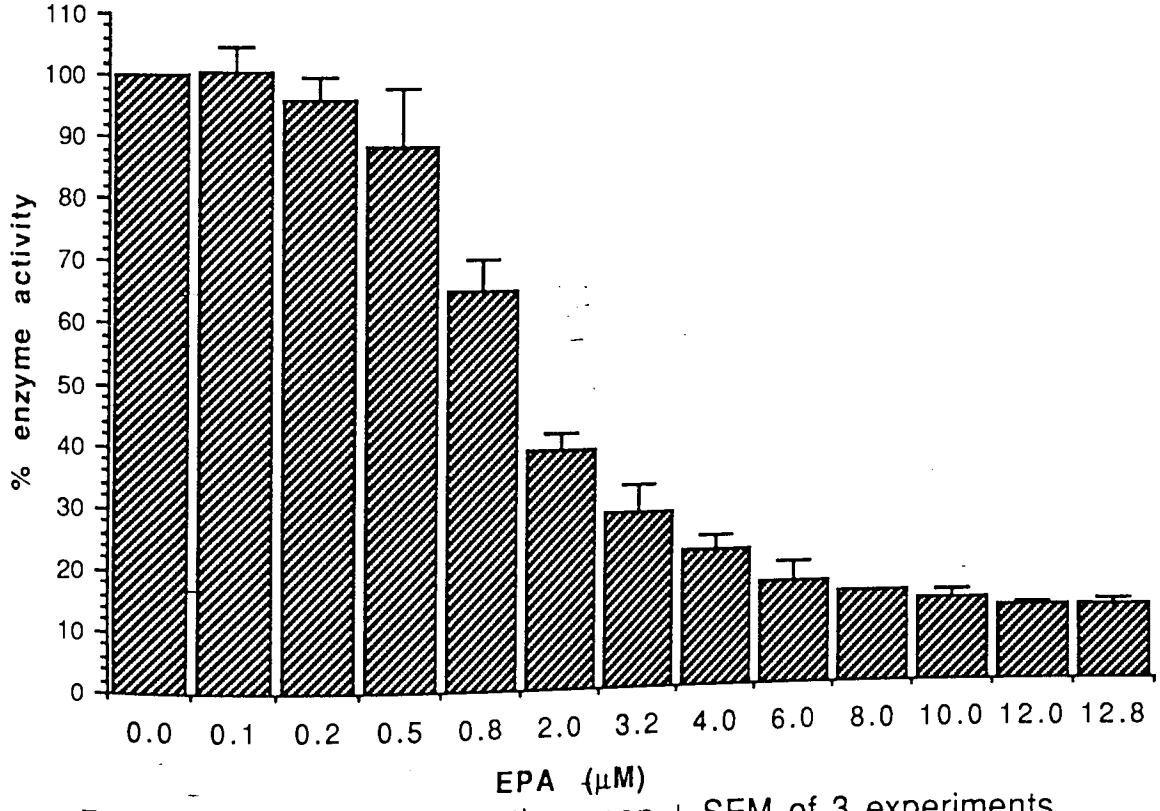


Figure 30B.

The inhibition of guanidinobenzoatase by EPA dissolved in 0.1M sodium carbonate.



Results are expressed as the mean ± SEM of 3 experiments.

Figure 31A.

The inhibition of guanidinobenzoatase by EPA.

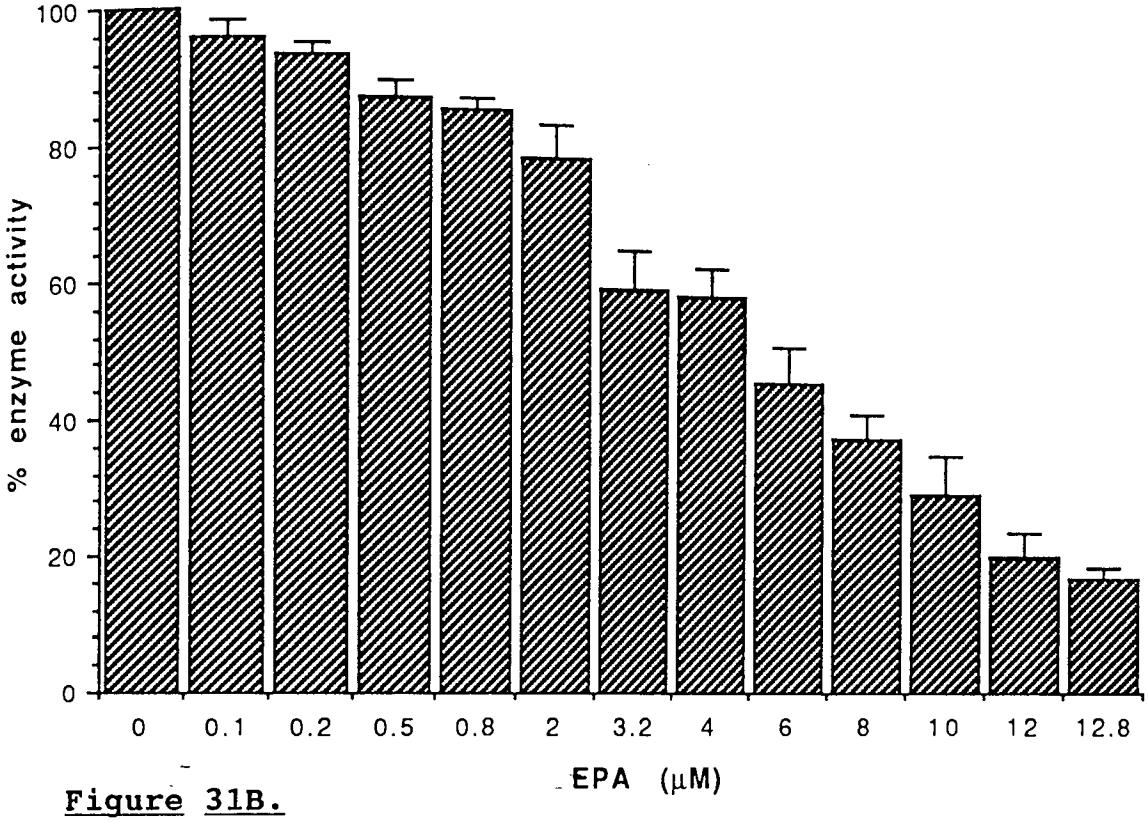
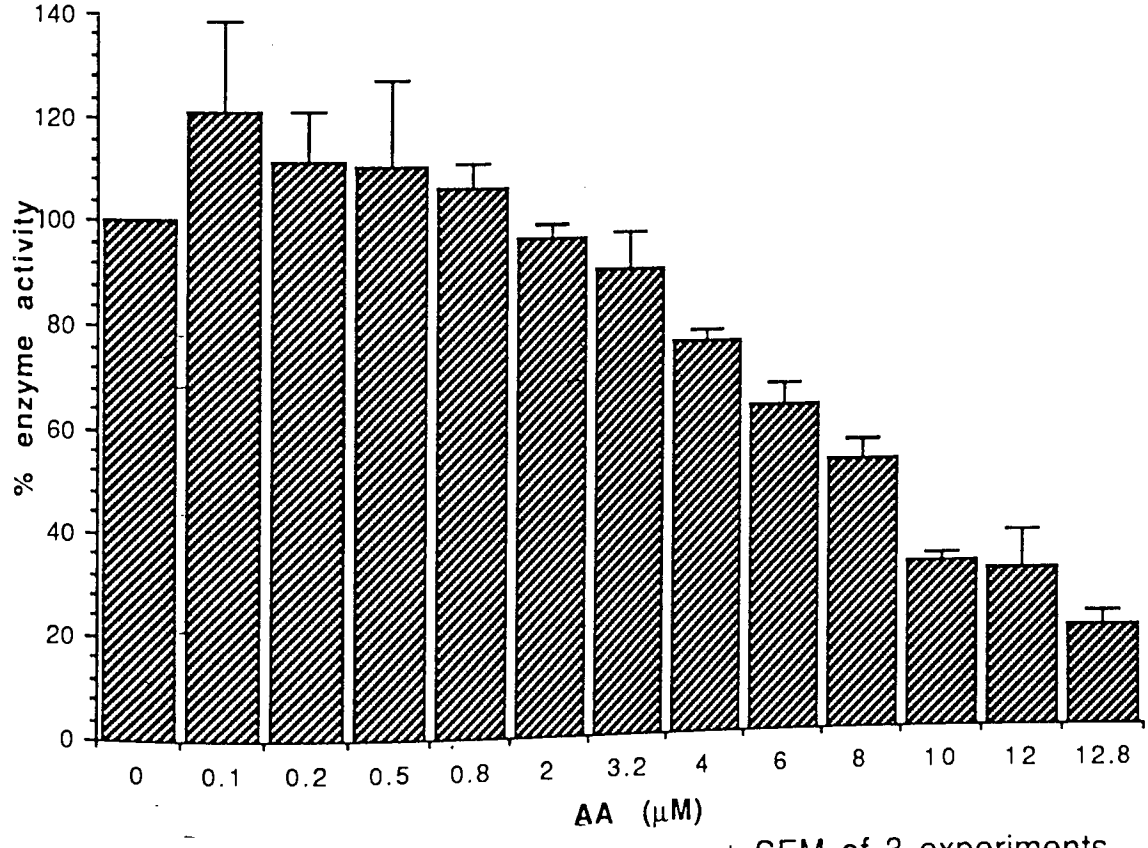


Figure 31B.

The inhibition of guanidinobenzoatase by AA.



Results are expressed as the mean ± SEM of 3 experiments.

Figure 32A.

The inhibition of guanidinobenzoatase by DHA.

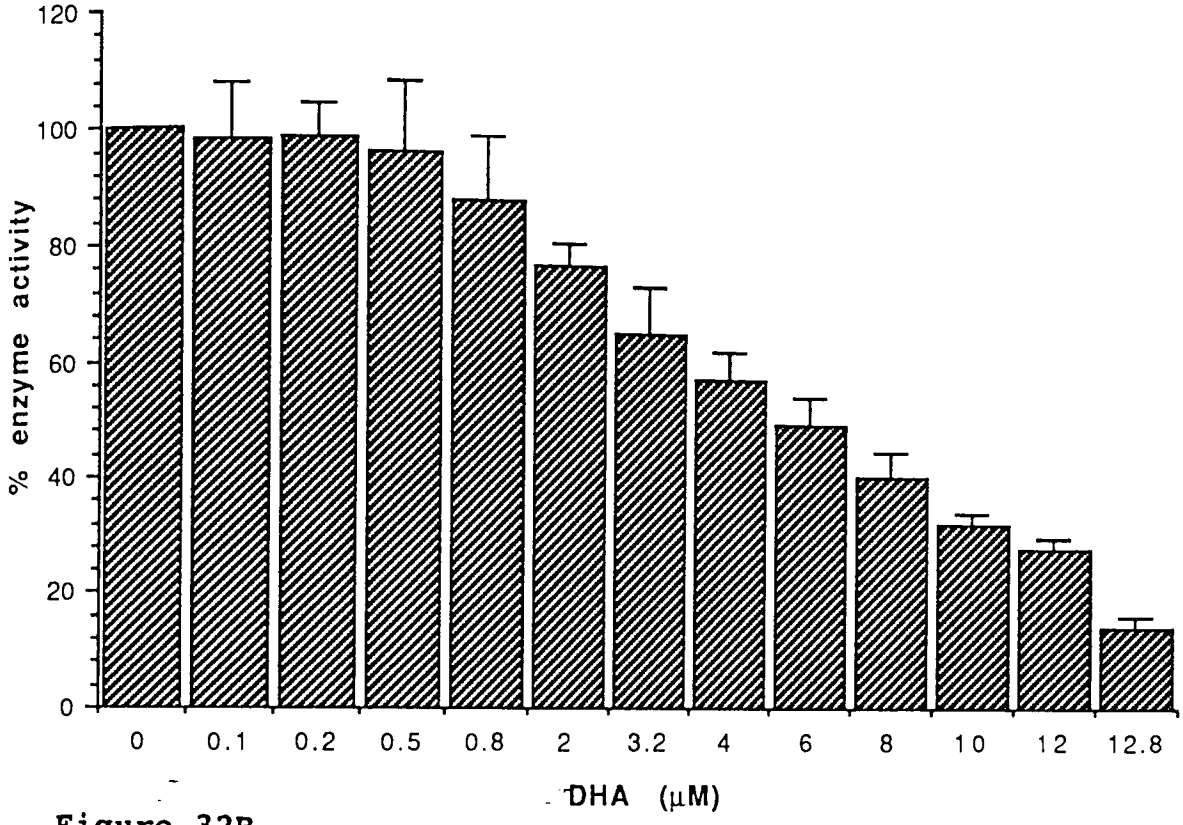
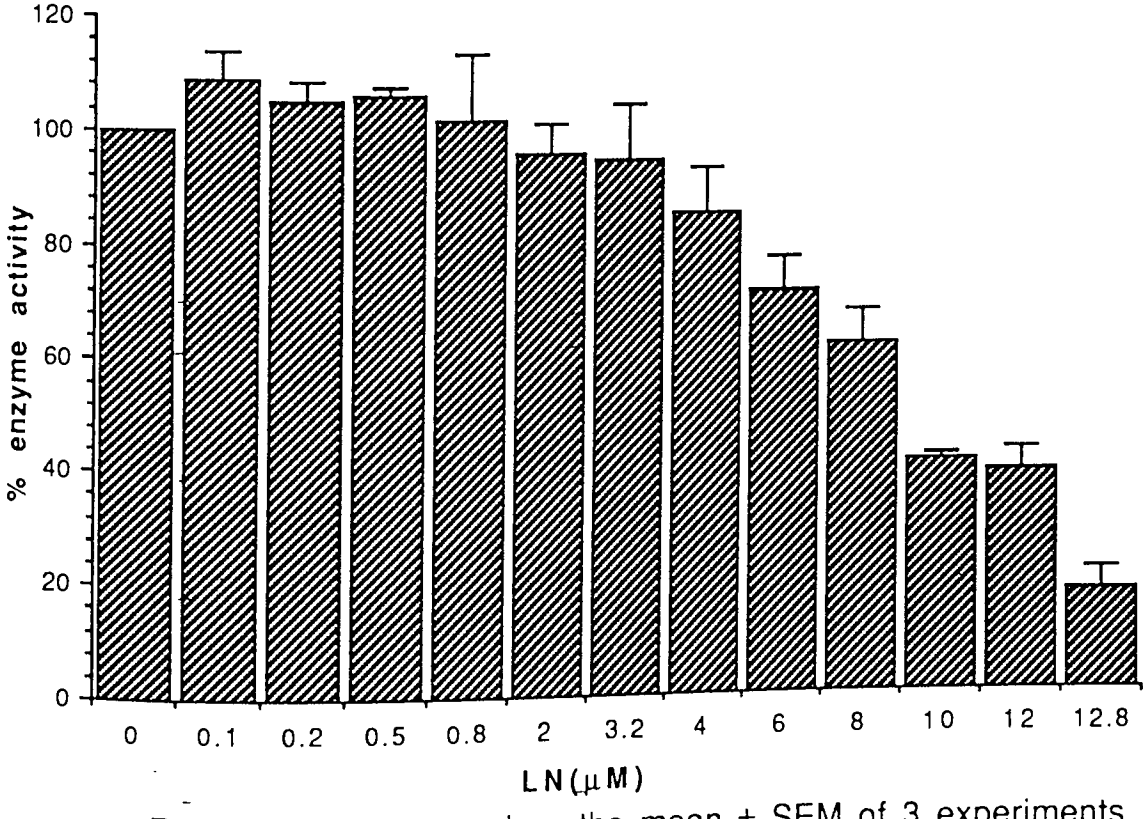


Figure 32B.

The inhibition of guanidinobenzoatase by LN.



Results are expressed as the mean ± SEM of 3 experiments.

Figure 33A.

The inhibition of guanidinobenzoatase by linoleic acid.

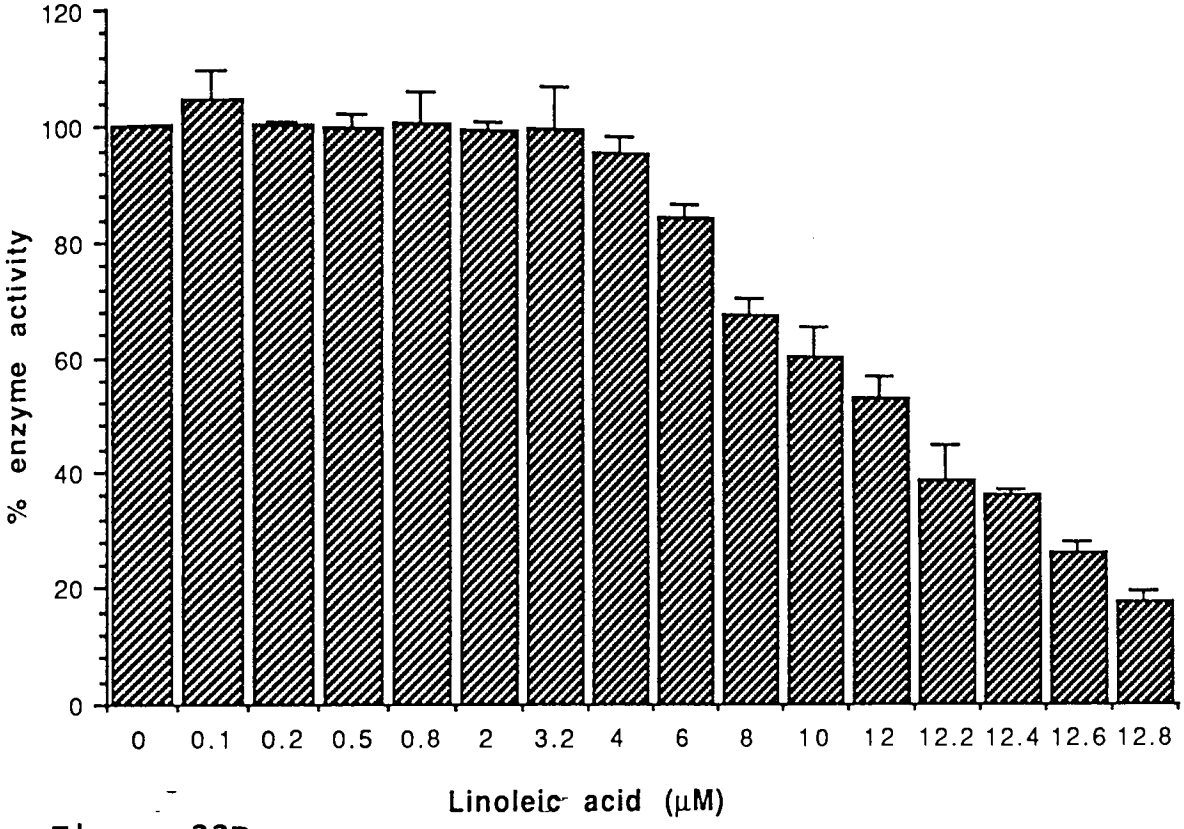
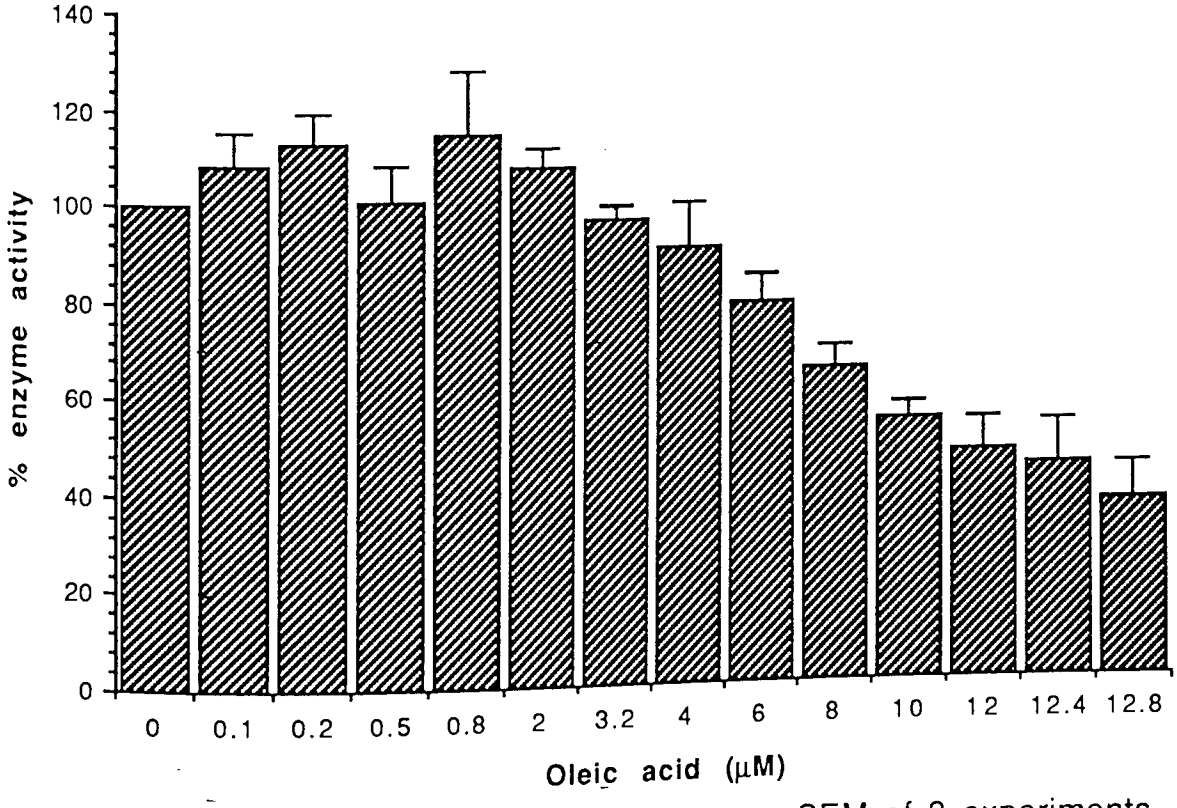


Figure 33B.

The inhibition of guanidinobenzoatase by oleic acid.



Results are expressed as the mean ± SEM of 3 experiments.

Figure 34A.

The inhibition of guanidinobenzoatase by BZAR.

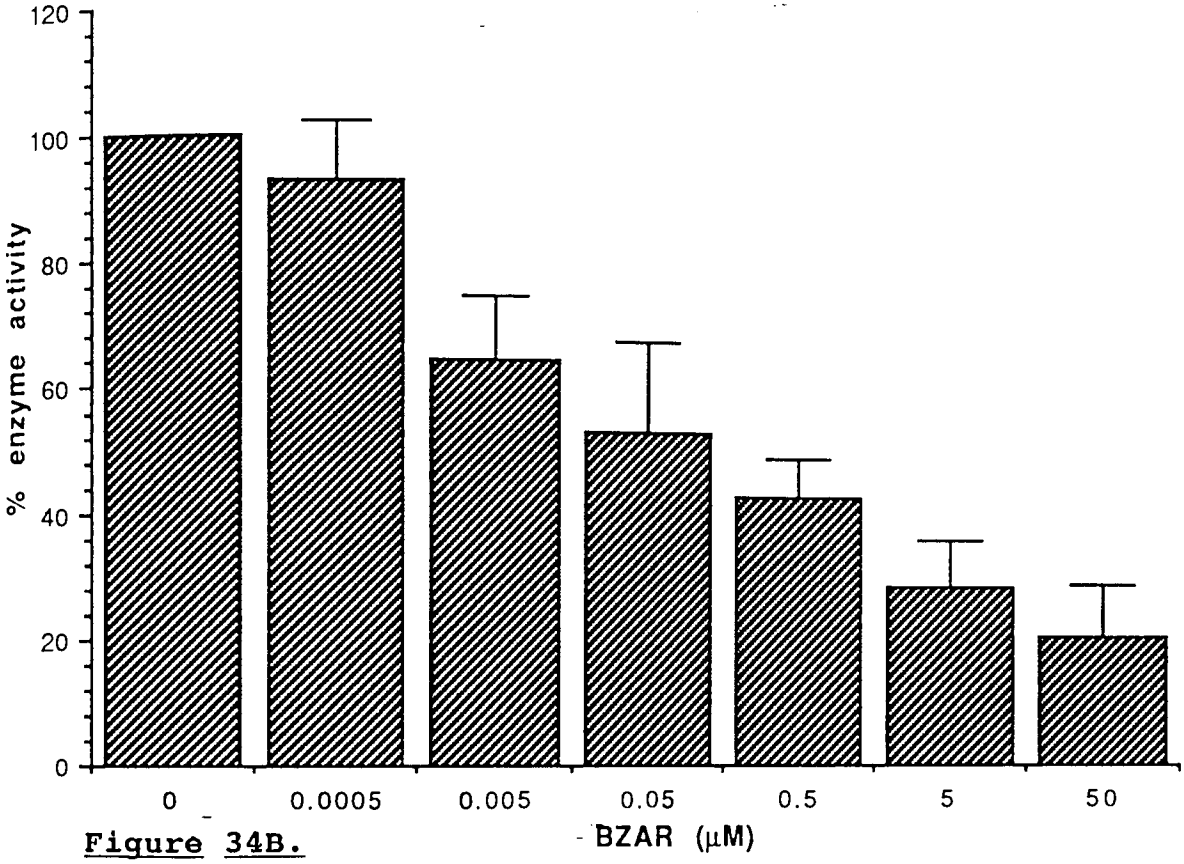
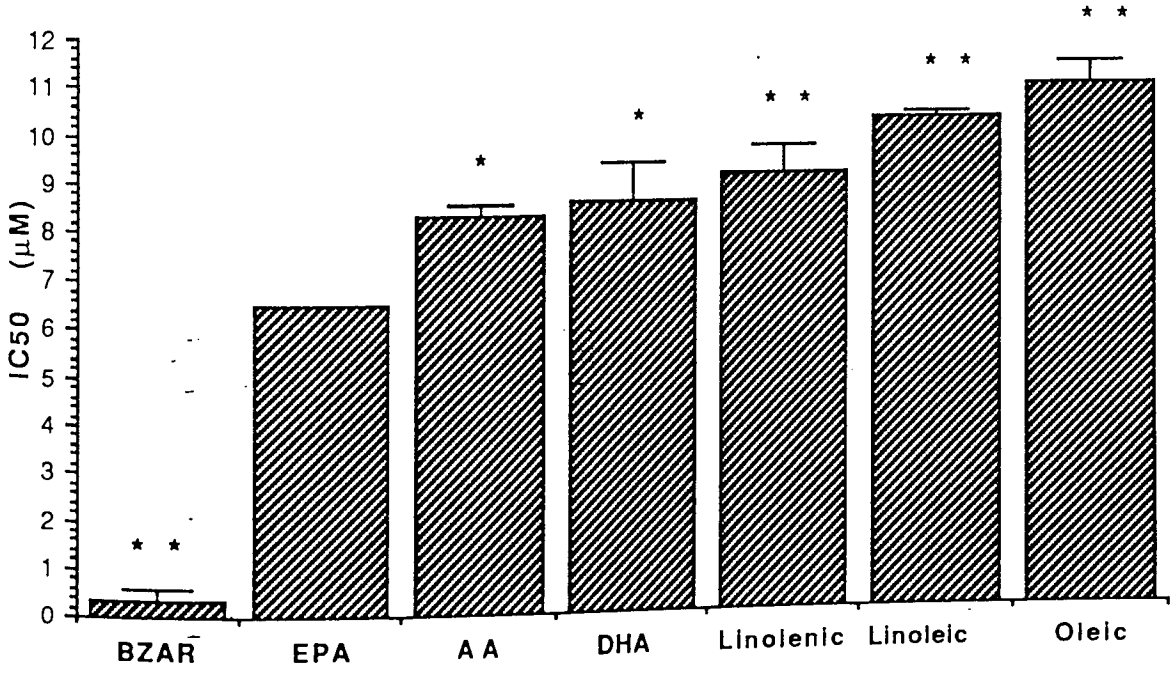


Figure 34B.

The IC50 values for the inhibitors of guanidinobenzoatase.



Results are expressed as the mean ± SEM of 3 experiments. Differences were determined by one-way ANOVAR as *=P<0.05, **=P<0.01 compared with EPA. EPA = eicosapentaenoic acid, AA = arachidonic acid, DHA = docosahexaenoic acid.

Figure 35A.

A Lineweaver Burke plot of the inhibition of guanidinobenzoatase by EPA.

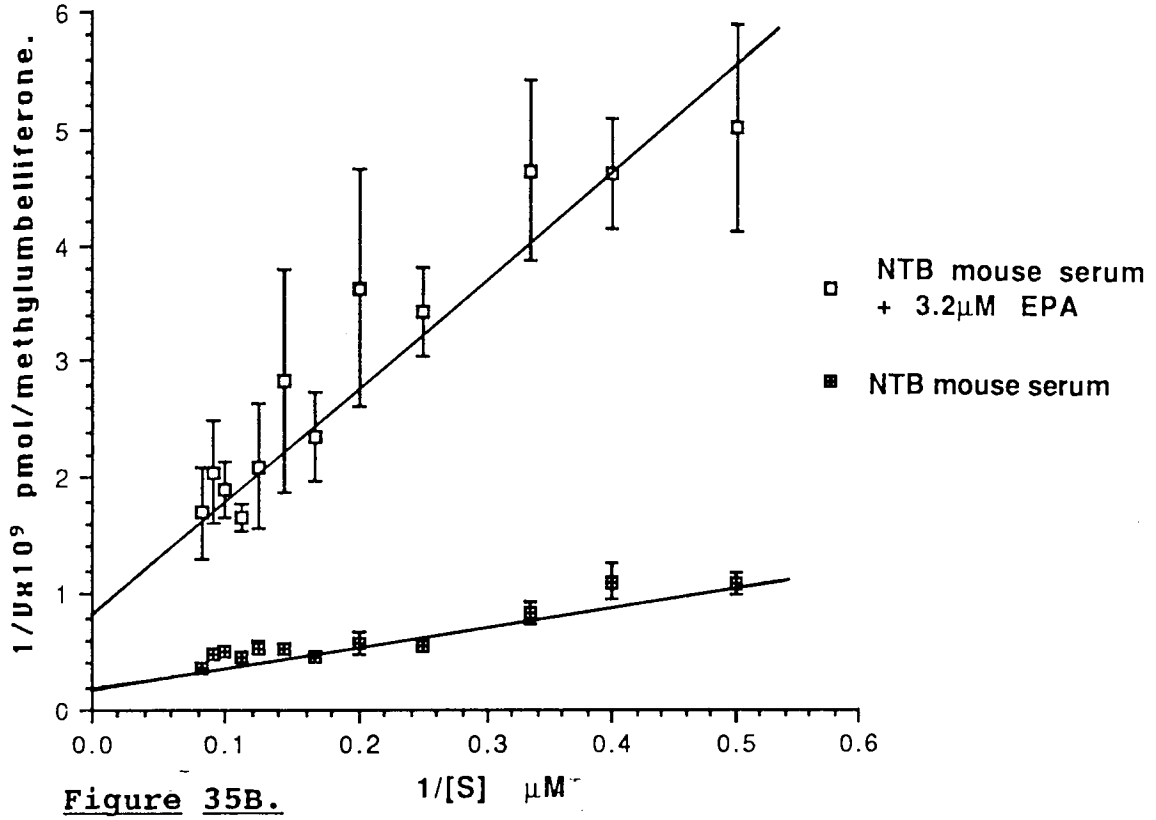
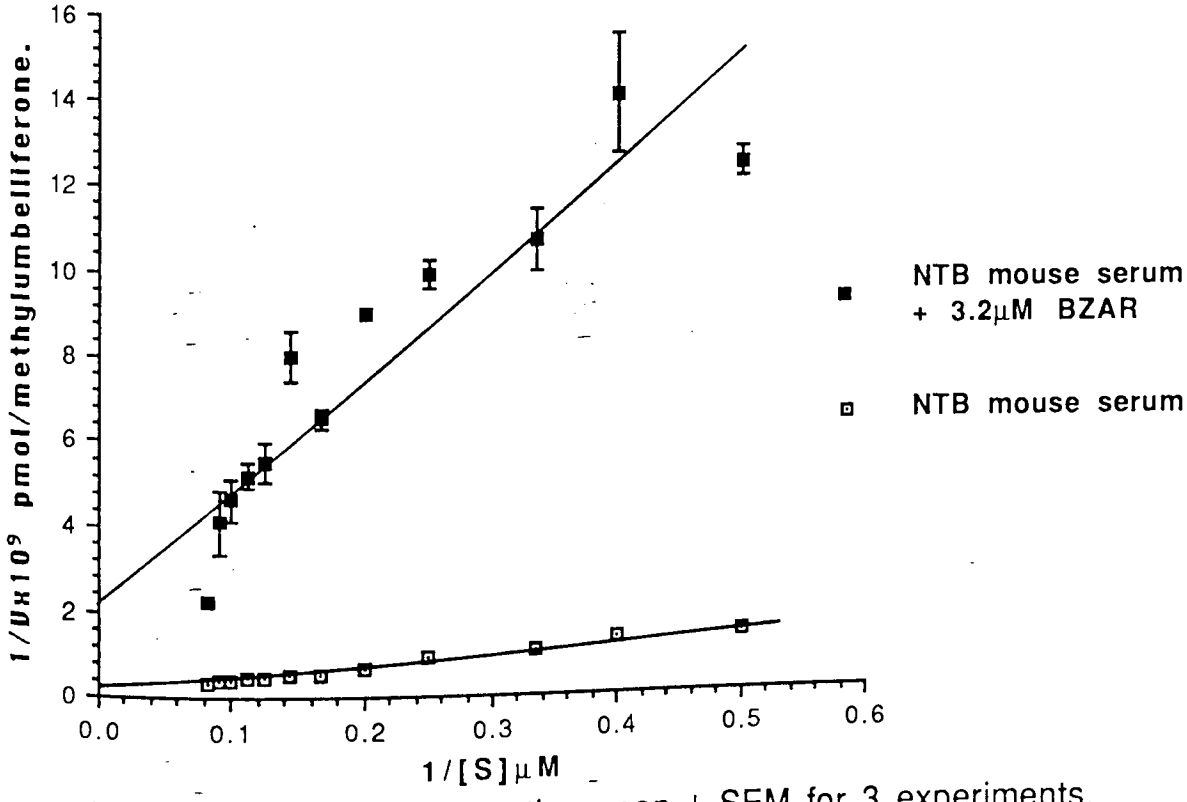


Figure 35B.

A Lineweaver Burke plot of the inhibition of guanidinobenzoatase by BZAR.



Results are expressed as the mean ± SEM for 3 experiments.

(Fig.34A) a known inhibitor of the enzyme (Steven et al, 1988). A comparison is shown in Fig.34B of the IC50 values for BZAR and the fatty acids. EPA was a significantly more potent inhibitor of guanidinobenzoatase than the other fatty acids. The inhibition of guanidinobenzoatase by BZAR is presented in the form of a Lineweaver-Burke plot in Fig. 35A and 35B. Both BZAR and EPA were non-competitive inhibitors of the enzyme with Ki values of 0.29 $\pm$ 0.02μM (mean $\pm$ SEM) and 0.98 $\pm$ 0.07μM respectively. The Ki value for BZAR is in the range for that previously reported by Steven et al (1988), and the Ki values for both inhibitors are lower than the Km value for guanidinobenzoatase (10.9 $\pm$ 1.4μM). At high concentrations (10^{-5}M) BZAR appeared to inhibit trypsin (Fig. 36A). However, this is probably a result of quenching caused by large amounts of red fluorescence produced by BZAR acting as a competitive substrate. BZAR was a profound inhibitor of esterase and tissue-type plasminogen activator (Fig. 36A, 36B). In contrast no inhibition of trypsin, esterase or tissue-type plasminogen activator was observed with EPA at concentrations up to 12.8μM (Fig. 37, 38A and 38B). Since tissue-type plasminogen activator is inhibited by ethanol, EPA was dissolved in either equimolar lysine or 0.1M sodium carbonate for this experiment. EPA dissolved in these solvents was an effective inhibitor of guanidinobenzoatase activity (Fig.30A and 30B). Since only guanidinobenzoatase is inhibited by EPA this suggests that this enzyme is

Figure 36A.

The effect of BZAR on esterase and trypsin.

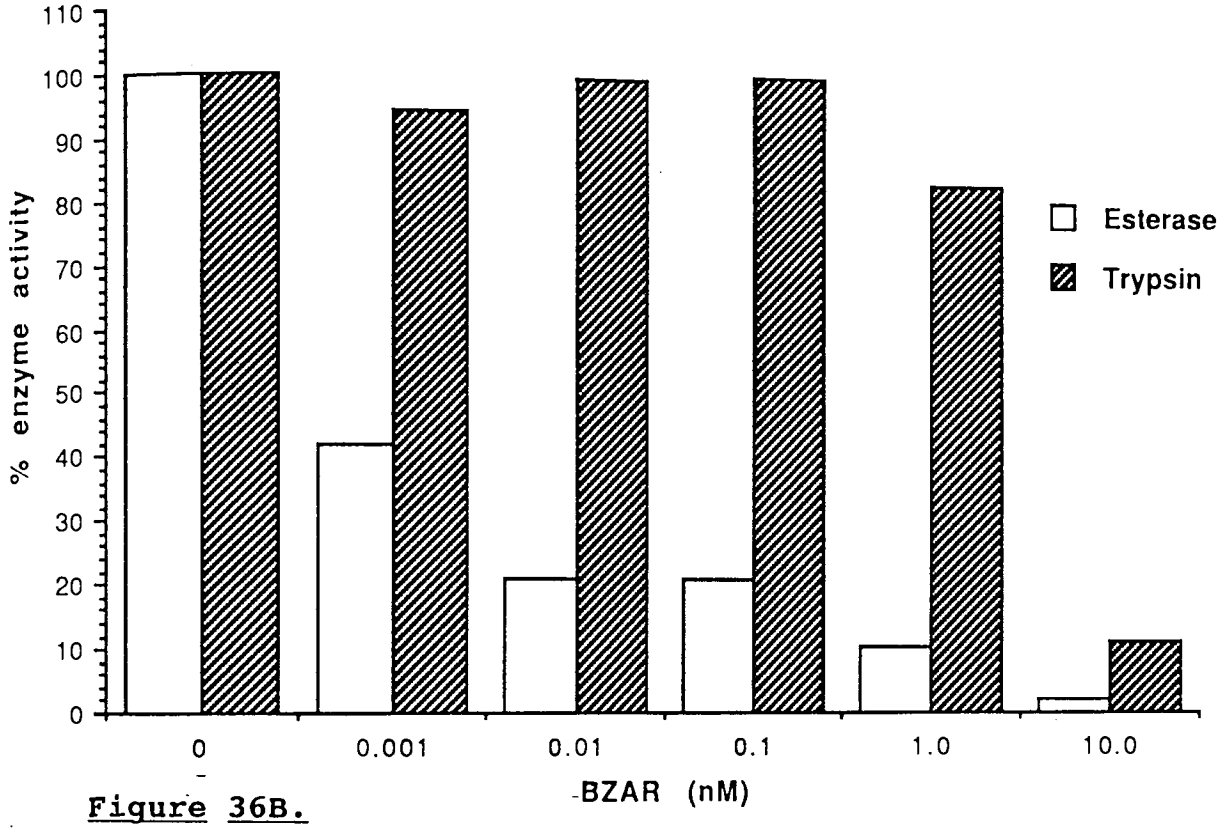
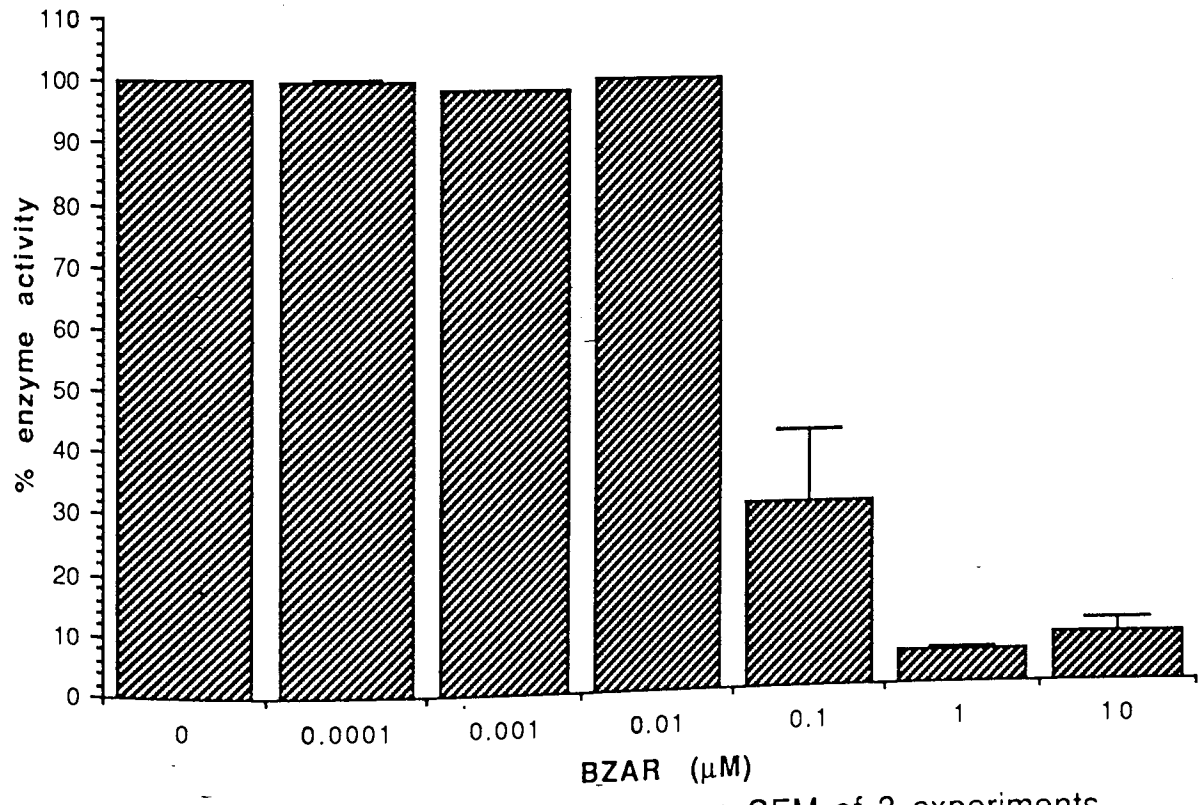


Figure 36B.

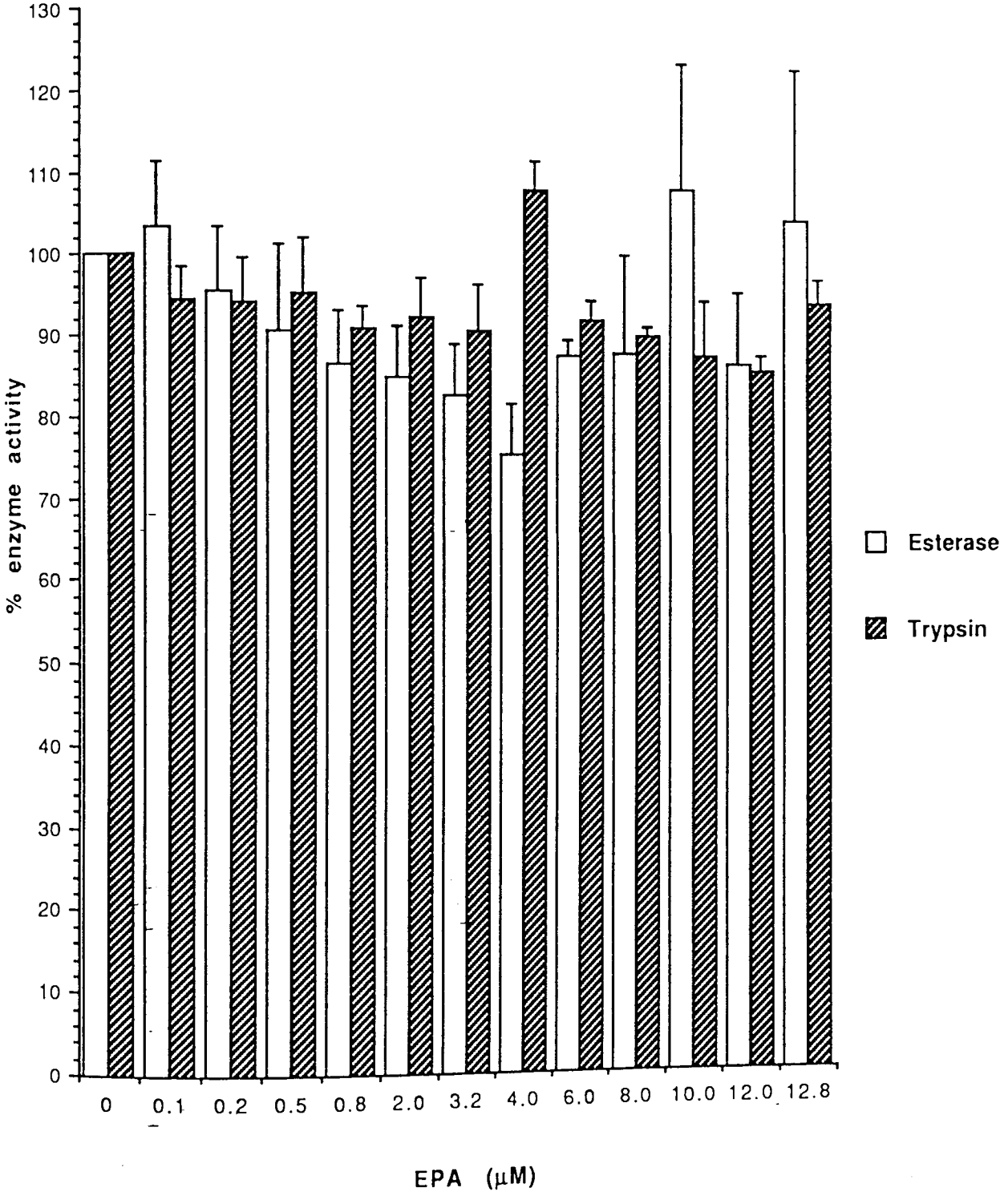
The effect of BZAR on tissue-type plasminogen activator.



Each bar represents the mean ± SEM of 3 experiments.

Figure 37.

The effect of EPA on esterase and trypsin.



Each bar represents the mean ± SEM for 3 experiments.

Figure 38A.

The effect of EPA dissolved in 0.1M sodium carbonate on tissue-type plasminogen activator.

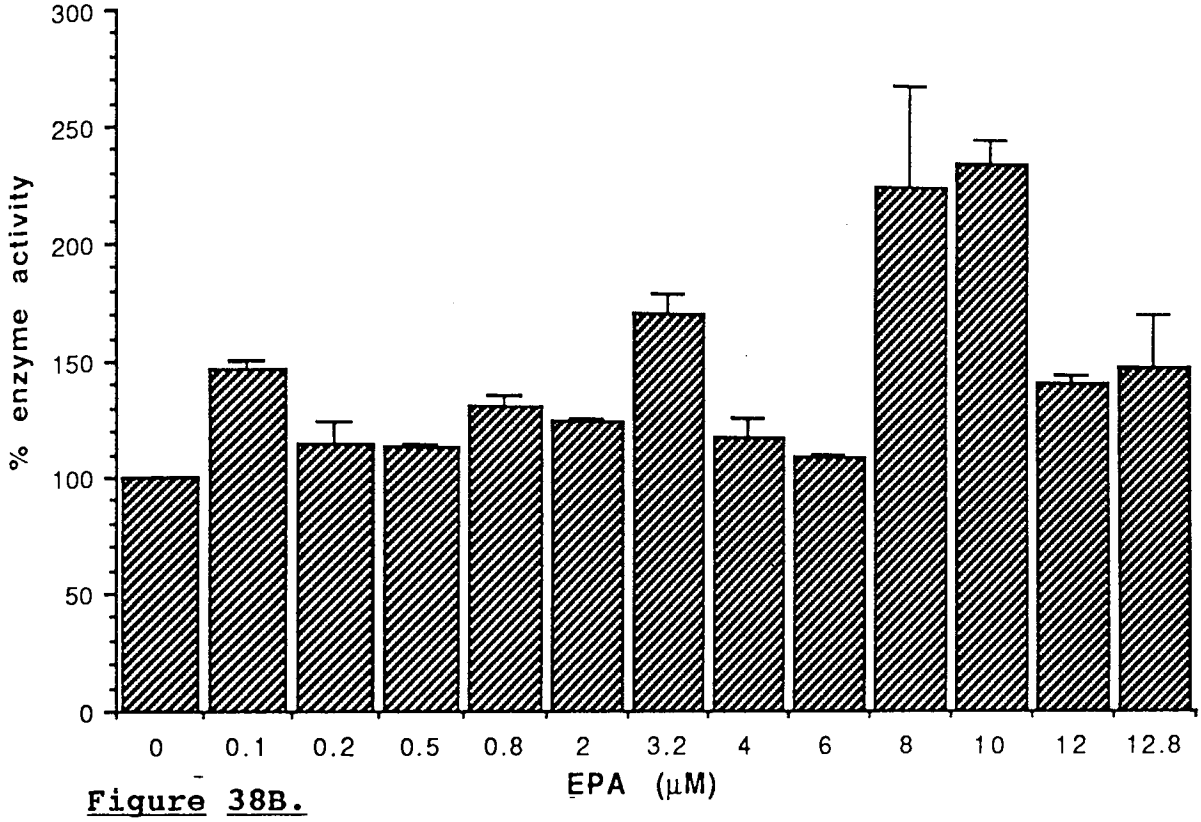
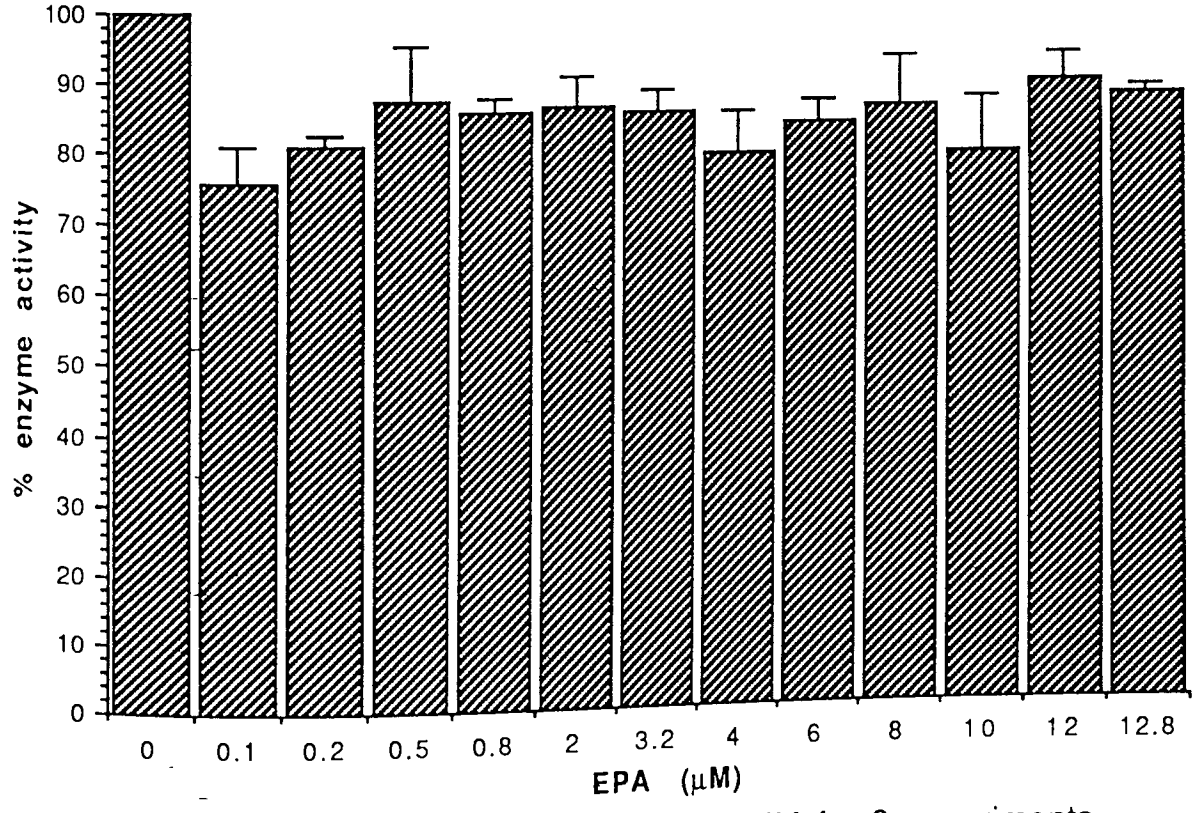


Figure 38B.

The effect of EPA dissolved in equimolar lysine on tissue-type plasminogen activator.



Each bar represents the mean ± SEM for 3 experiments.

Figure 39A.

The effect of a synthetic plasminogen activator inhibitor on guanidinobenzoatase.

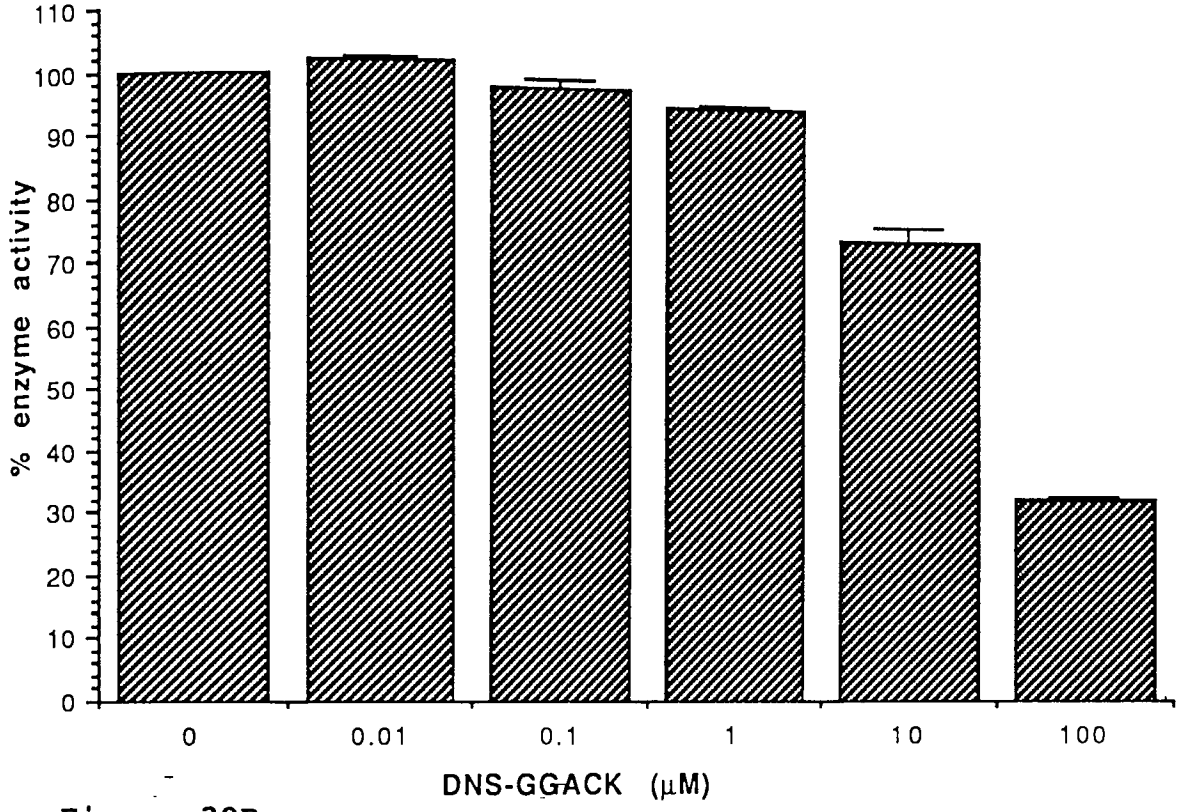
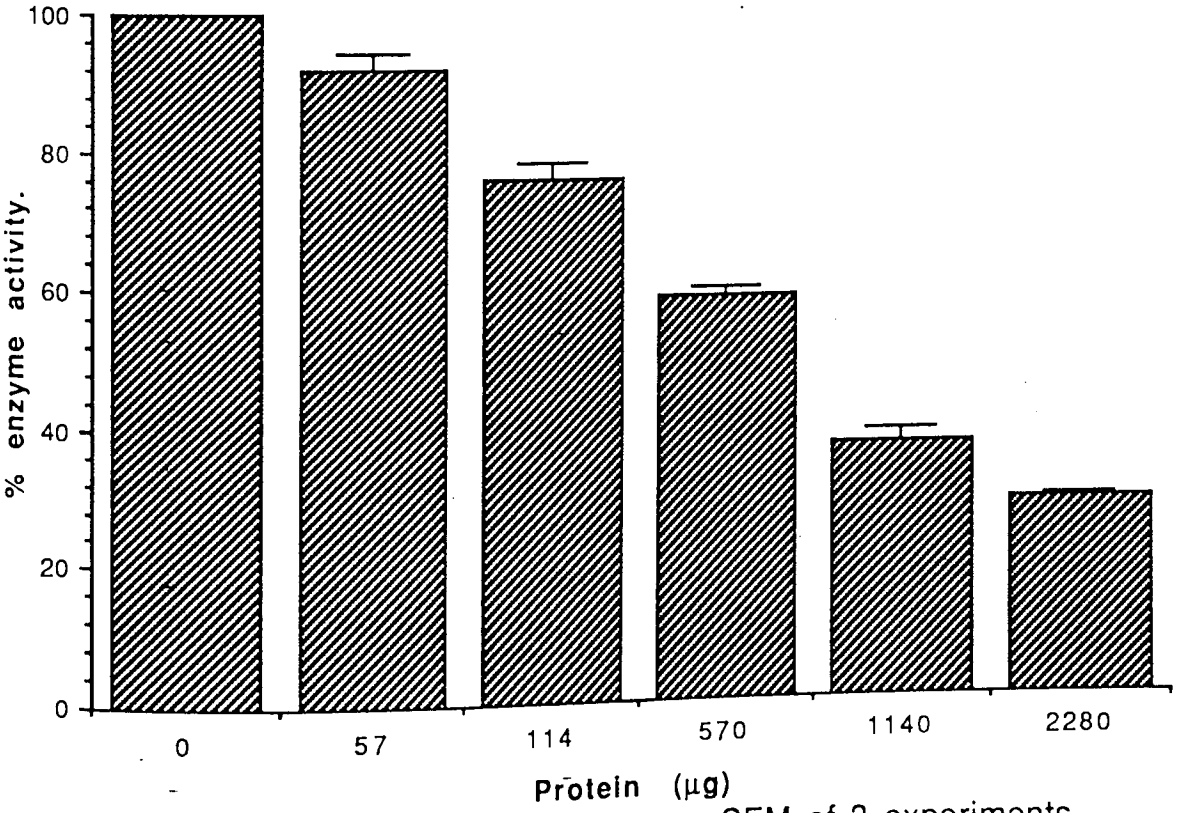


Figure 39B.

The effect of isotonic extract of MAC16 tumour on guanidinobenzoatase.



Each bar represents the mean ± SEM of 3 experiments.

distinct from trypsin, esterase or tissue-type plasminogen activator. Guanidinobenzoatase was inhibited at high concentrations (10-100μM) by dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone (DNS-GGACK) (Fig. 39A) - a synthetic peptide derivative plasminogen activator inhibitor. Similarly an isotonic extract of MAC16 tumour demonstrated marked guanidinobenzoatase inhibition (Fig.39B). These results suggest conversely that guanidinobenzoatase and tissue-type plasminogen activator share some homologies. The guanidinobenzoatase inhibitors present in tumour extract have been shown to be exchangeable with purified inhibitor of plasminogen activator on fixed tumour cell surfaces (Steven and Booth, 1991).

Formaldehyde fixed wax embedded sections, after dewaxing, were stained with 9-aminoacridine. The cell surfaces of the MAC16 cells bound 9-aminoacridine and fluoresced yellow (Fig.40A and 40B), demonstrating the presence of active guanidinobenzoatase on these cells. Pretreatment of the sections with EPA ($3.7x10^{-4}$M) completely blocked the binding of 9-aminoacridine to the guanidinobenzoatase on the surface of these tumour cells (Fig. 41A). The interaction of EPA with the cell surface guanidinobenzoatase was shown to be reversible, since the EPA could be washed out of the cell surface guanidinobenzoatase by placing the slide in a tank of

Figure 40A.

A fluorescent micrograph of a wax section of MAC16 tumour stained with 9-aminoacridine for 2 min. Magnification X25.

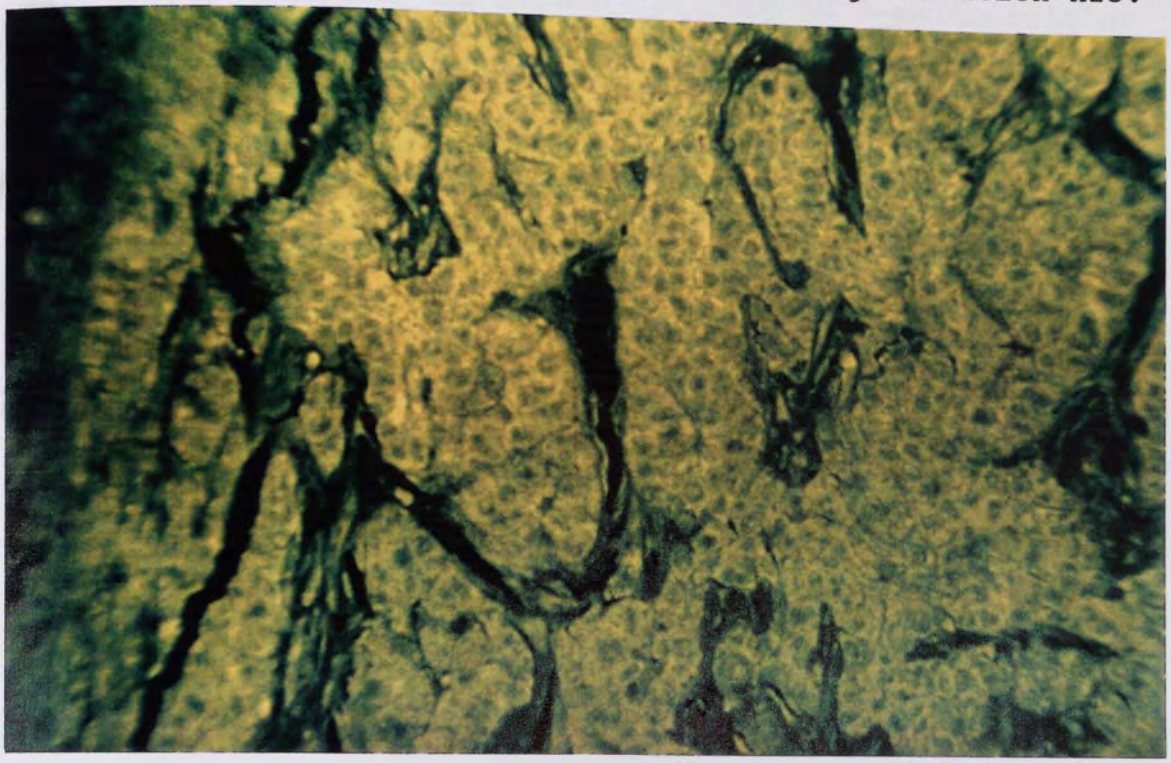
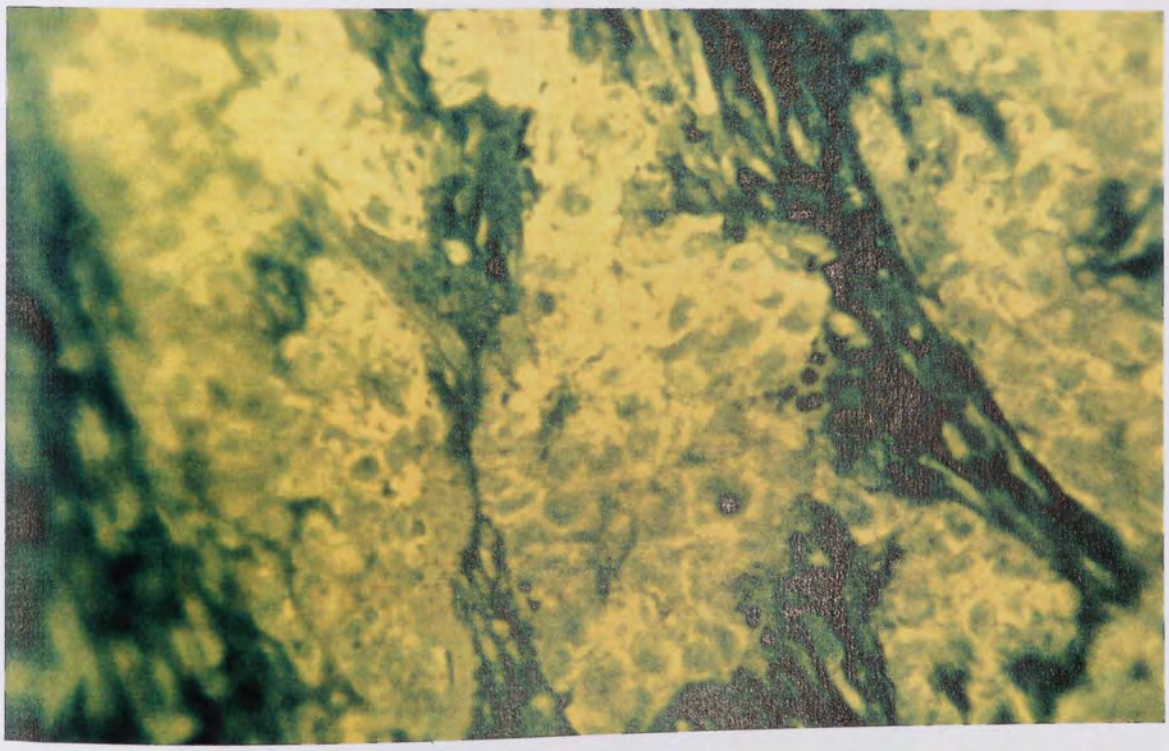


Figure 40B.

A fluorescent micrograph of a wax section of MAC16 tumour stained with 9-aminoacridine for 2 min. Magnification X50.



Tumour cells possessing guanidinobenzoatase activity appear yellow. The background of connective tissue, intracellular matrix and inactive cells appear green.

Figure 41A.

A fluorescent micrograph of a wax section of MAC16 tumour - pre-treated with EPA for 30 min followed by 9-aminoacridine staining for 2 min. Magnification X25.

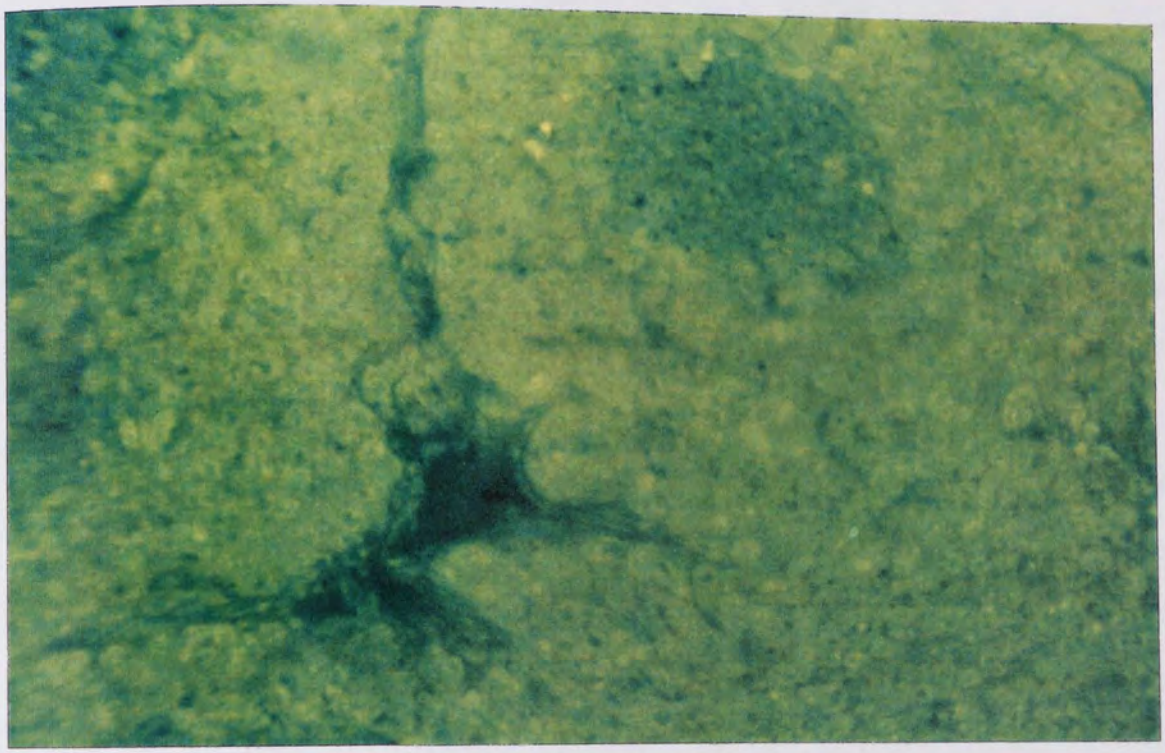
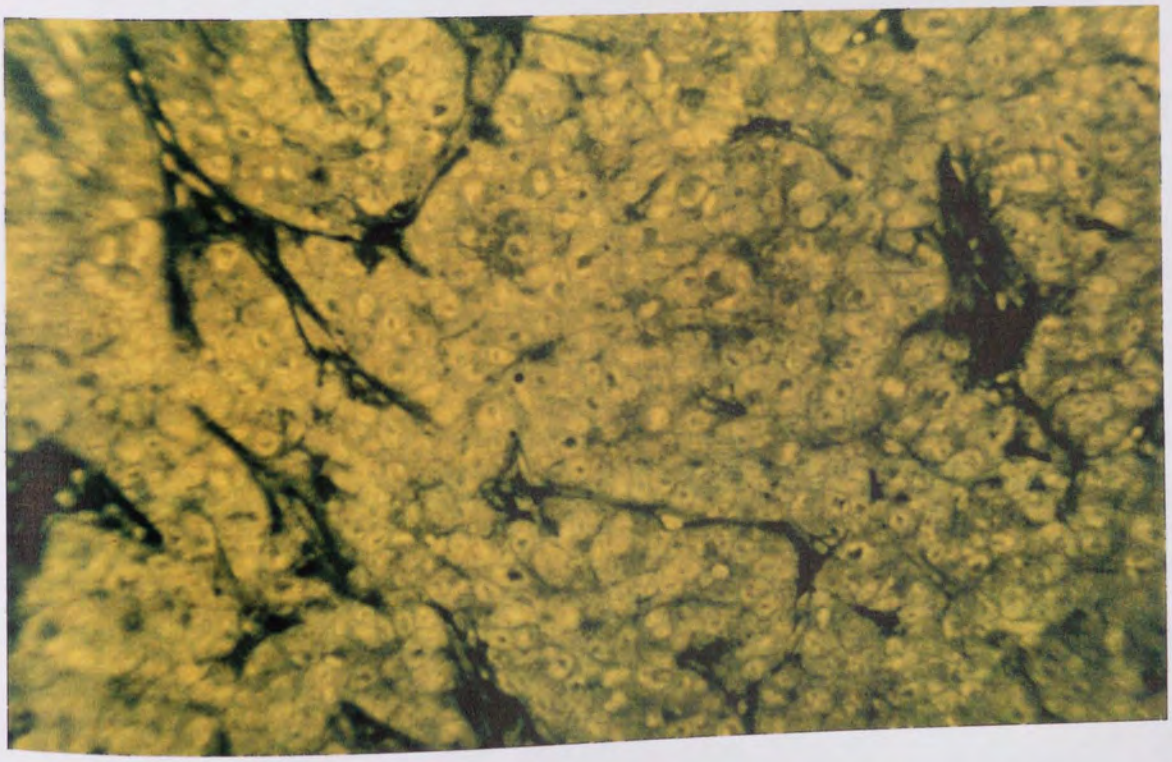


Figure 41B.

A fluorescent micrograph of a wax section of MAC16 tumour - pretreated with EPA for 30 min followed by 9-aminoacridine staining for 1h in the absence of EPA. Magnification X25.



9-aminoacridine (300ml, 10^{-3}M for 1h). In this case the concentration of 9-aminoacridine was maintained whilst the concentration of EPA in the 300ml of washing fluid was diluted sufficiently to be considered minimal (Fig. 41B). Pretreatment of dewaxed MAC16 tumour sections with mouse serum for 1h resulted in the inhibition of the cell surface guanidinobenzoatase as judged by the ability of the cells to bind 9-aminoacridine (data similar to Fig. 41A). Surface bound guanidinobenzoatase and guanidinobenzoatase-inhibitor complex were removed from the MAC16 tumour cells by exposure to fibrin films with the result that the MAC16 cells failed to bind 9-aminoacridine and did not fluoresce yellow, even after formaldehyde treatment. The inhibitor released from the guanidinobenzoatase-inhibitor complex was shown to be transferrable and capable of inhibiting further sections of enzymically active MAC16 tumour. Fibrin films have been shown to bind strongly to tissue-type plasminogen activator forming a new enzyme-fibrin complex capable of cleaving plasminogen (Steven and Blakey, 1992).

4.4.2. Discussion.

The enhancement of guanidinobenzoatase activity seen with trypsin inhibitor demonstrates that trypsin and guanidinobenzoatase are not synonymous enzymes. The potentiating effect seen is probably due to the inhibition

of enzymes degrading guanidinobenzoatase. The fluorogenic substrate for trypsin-like enzymes BZAR, has previously been shown to be a potent inhibitor of guanidinobenzoatase, both in solution and on the surface of tumour cells (Steven et al, 1988a). BZAR is bound non-competitively to the active centre of guanidinobenzoatase by reason of its containing a strongly protonated amino group simulating the arginine residue, which is the preferred cleavage point in synthetic peptides (Steven et al, 1985). Thus the ability of polyunsaturated fatty acids, in particular EPA, to inhibit the enzyme was somewhat surprising, in view of the lack of structural similarity to known inhibitors of the enzyme. The ability of EPA to block the binding of 9-aminoacridine in wax embedded sections of tumour, suggested either that it occupied the same binding site on the enzyme, viz the active centre, or that it bound to a more distant site, which induced a conformational change in the enzyme, preventing the binding of 9-aminoacridine. Unlike BZAR the binding of EPA to the enzyme was reversed by competition with 9-aminoacridine, suggesting a reversible non-competitive binding of EPA to guanidinobenzoatase. The inhibitory effect appears to be very specific to guanidinobenzoatase, since other similar proteolytic enzymes, trypsin, esterase and tissue-type plasminogen activator were unaffected by EPA suggesting that the effect does not arise solely from the detergent properties of the polyunsaturated fatty acids. In contrast

BZAR was shown to be an effective inhibitor of esterase and tissue-type plasminogen activator. It is unusual for a peptide (BZAR) to inhibit an esterase. However, Liu et al (1980) have demonstrated the active site titration of the esterase activity of plasmin using a fluorescein derivative 3',6'-bis(4-guanidinobenzoyloxy)-5-(N'-4-carboxylphenyl) thioureidospiro[isobenzofuran-1(3H),9'-[9H]xanthen)-3-one - commonly known as FluoresceinDiEster (FDE). FDE and BZAR share a number of structural homologies - the most important being, in this case, the possession of two ester bonds. BZAR exhibits negligible intrinsic fluorescence, because the amino groups of rhodamine are blocked by acetylation - consequently the intensely coloured dye is converted to a colourless, non-fluorescent form. The cleavage of a single amide bond converts the non-fluorescent bisamide substrate into a highly fluorescent monoamide product (Leytus et al, 1983). If esterase is capable of cleaving BZAR at the amide ester linkage the product will not fluoresce and interfere with the assay. Thus it is proposed that BZAR inhibits esterase by successfully competing with o-nitrophenol butyrate for the active site. BZAR is cleaved by trypsin at the amide bonds to yield the red fluorophore rhodamine - this fluorescence quenches the detection of 7-amido-4-methylcourmarin and gives the impression of inhibition.

There is controversy over the relationship between soluble and cell-bound guanidinobenzoatase and tissue-type plasminogen activator. The soluble mouse serum enzyme cannot be the same as bound enzyme for two reasons - firstly MAC16 cell surface guanidinobenzoatase was inhibited by exposure to mouse serum for an hour, clearly in contrast to the soluble enzyme, which was active in mouse serum, and secondly the MAC16 guanidinobenzoatase was transferred to and bound to fibrin. Soluble guanidinobenzoatase was contained in mouse serum prepared from clotted blood - consequently if the two enzymes were identical the guanidinobenzoatase would have been removed in the clot and there would be no enzyme activity. If soluble and cell-bound guanidinobenzoatase were tissue-type plasminogen activator EPA would be expected to inhibit tissue-type plasminogen activator in true solution. Conversely however, a synthetic tPA inhibitor and a crude extract of MAC16 tumour inhibited mouse serum guanidinobenzoatase. It could be argued that tumour extract caused destruction of guanidinobenzoatase rather than an inhibition of the enzyme. Steven et al (1988f) has demonstrated that cell-bound guanidinobenzoatase activity can be regained after formaldehyde displacement of tumour associated enzyme inhibitors. Thus guanidinobenzoatase is not irreversibly destroyed, but reversibly inhibited.

In summary - it is probable that cell-bound and soluble

guanidinobenzoatase are distinct enzymes with some common properties such as their ability to cleave guanidinobenzoates and to be inhibited by BZAR and EPA. Soluble guanidinobenzoatase is unlikely to be trypsin, an esterase or tissue-type plasminogen activator as they are not inhibited by EPA. Thus the soluble mouse serum enzyme appears in some respects to be more closely related to cell-bound guanidinobenzoatase than to other enzymes. It may be that the enzymes are related iso-enzymic forms. Since single tumour cells capable of metastasis possess uninhibited guanidinobenzoatase (Steven et al 1988c) inhibitors of this enzyme might also be expected to have an inhibitory effect on the metastatic process. Indeed culturing murine melanoma cells with pure EPA in ethanol has been shown to cause a dose and time-dependent decrease in invasiveness, collagenase IV production and a reduced ability to metastasize to the lung after i.v. injection (Reich et al 1989). The antimetastatic activity of BZAR also seems worthy of investigation.

4.5. Determination of the relationship between fatty acid structure and degree of guanidinobenzoatase inhibition - using molecular modelling.

4.5.1. Results.

The IC50 values for the inhibition of guanidinobenzoatase

by eicosapentaenoic (EPA), docosahexaenoic (DHA), arachidonic (AA), linolenic (LN), linoleic (L) and oleic (OL) acid are shown in Fig. 34B. The molecular models of the polyunsaturated fatty acids modelled on two comparative systems are shown in Fig. 42-47. The Charmm and Chemx models have dot surfaces coloured on Van de Waals and electrostatic energies respectively. Various parameters were measured on each model and these are expressed in table 2.

Table 2.

	IC50	**MW**	**No.double bonds.**	**Max. length.**	**Max. width.**	**Vol. $W^2xL/2$**
EPA	6.51	302.5	5	11.94	7.74	357.7
AA	8.27	304.5	4	12.19	8.30	419.8
DHA	8.54	328.5	6	10.44	9.61	482.1
LN	9.14	280.4	3	12.30	7.25	323.3
L	10.33	278.4	2	12.99	5.35	185.9
OL	10.66	282.5	1	21.82	1.54	25.9

The IC50 values are in uM and width, length and volume are in Angstroms ($x10^{-3}$).

In an attempt to equate a particular structural aspect of a molecule to its inhibitory effect a computerised model of regression analysis was used. This enabled the comparison of single or mutiple variables to the reciprocal of guanidinobenzoatase activity. There was no significant

Figure 42A.

A Charmm molecular model of eicosapentaenoic acid.

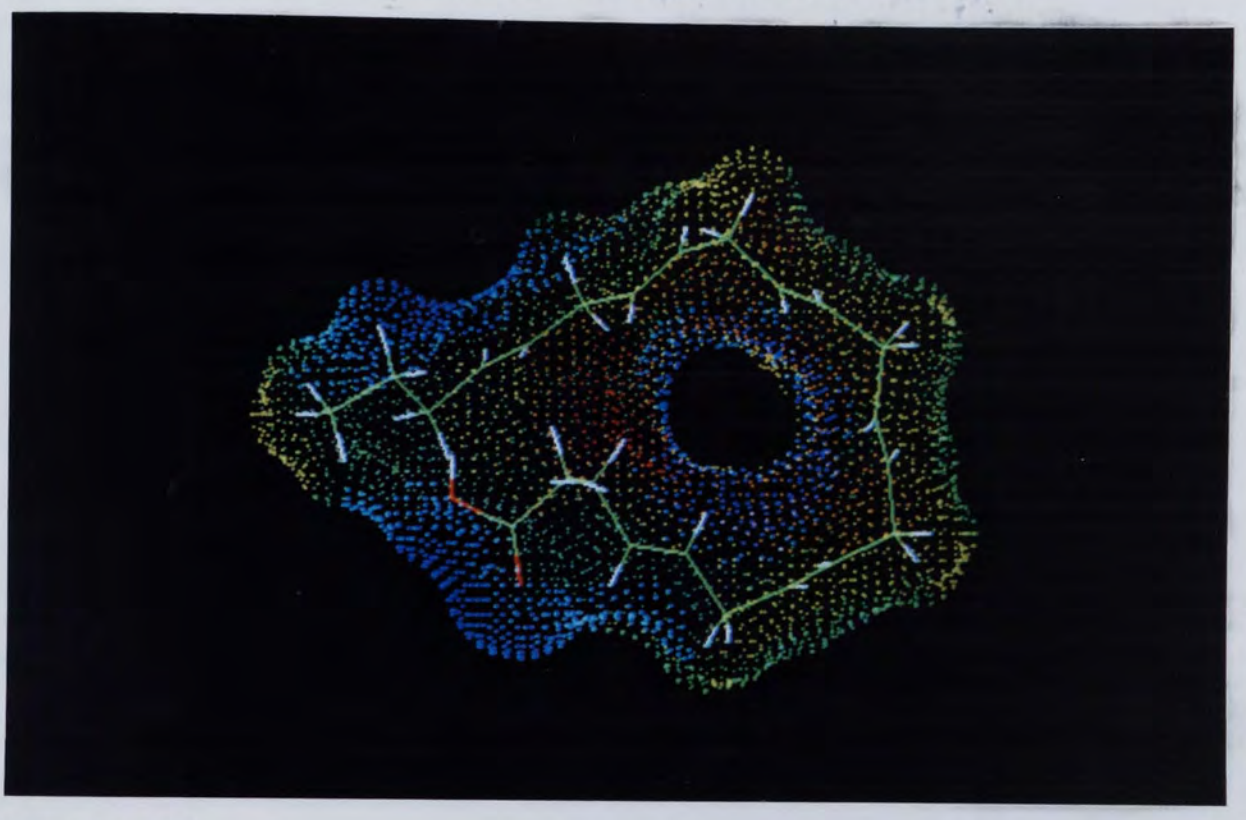


Figure 42B.

A Chemx molecular model of eicosapentaenoic acid.

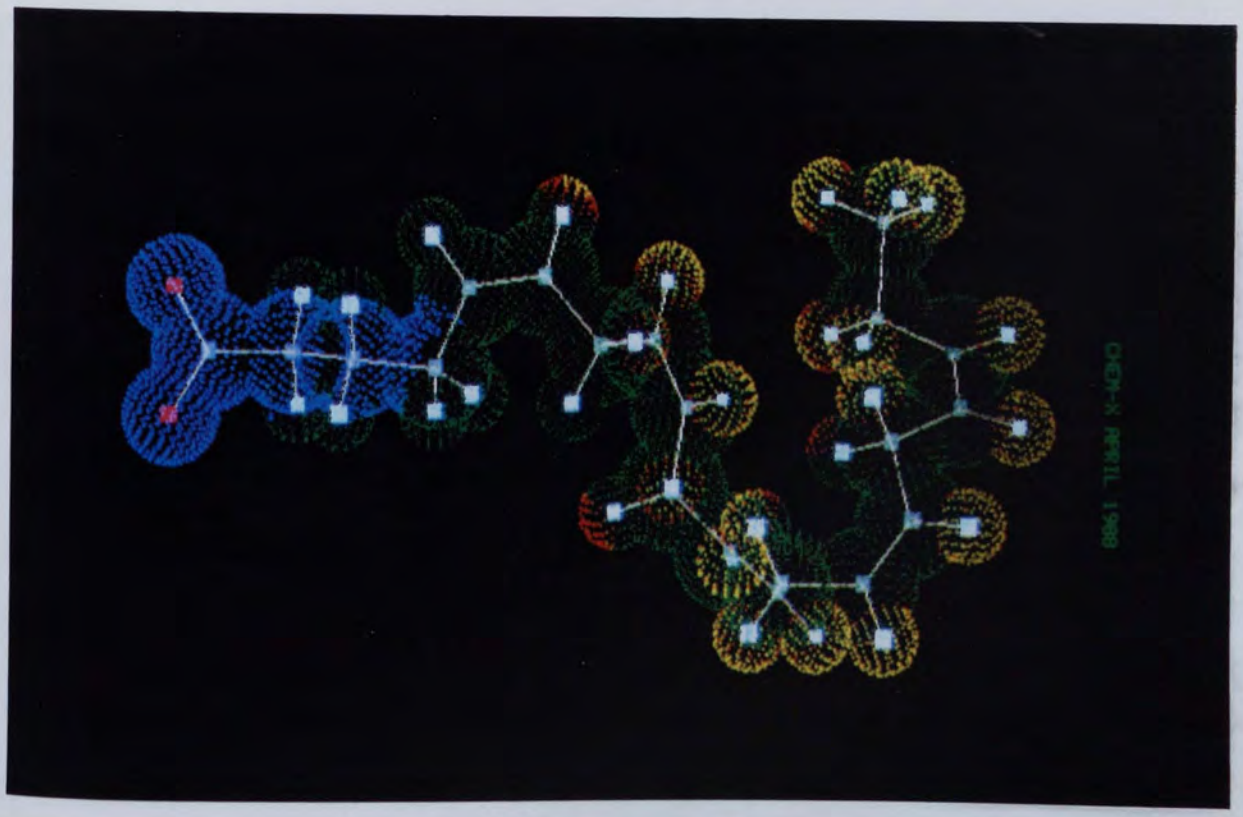


Figure 43A.

A Charmm molecular model of docosahexaenoic acid.

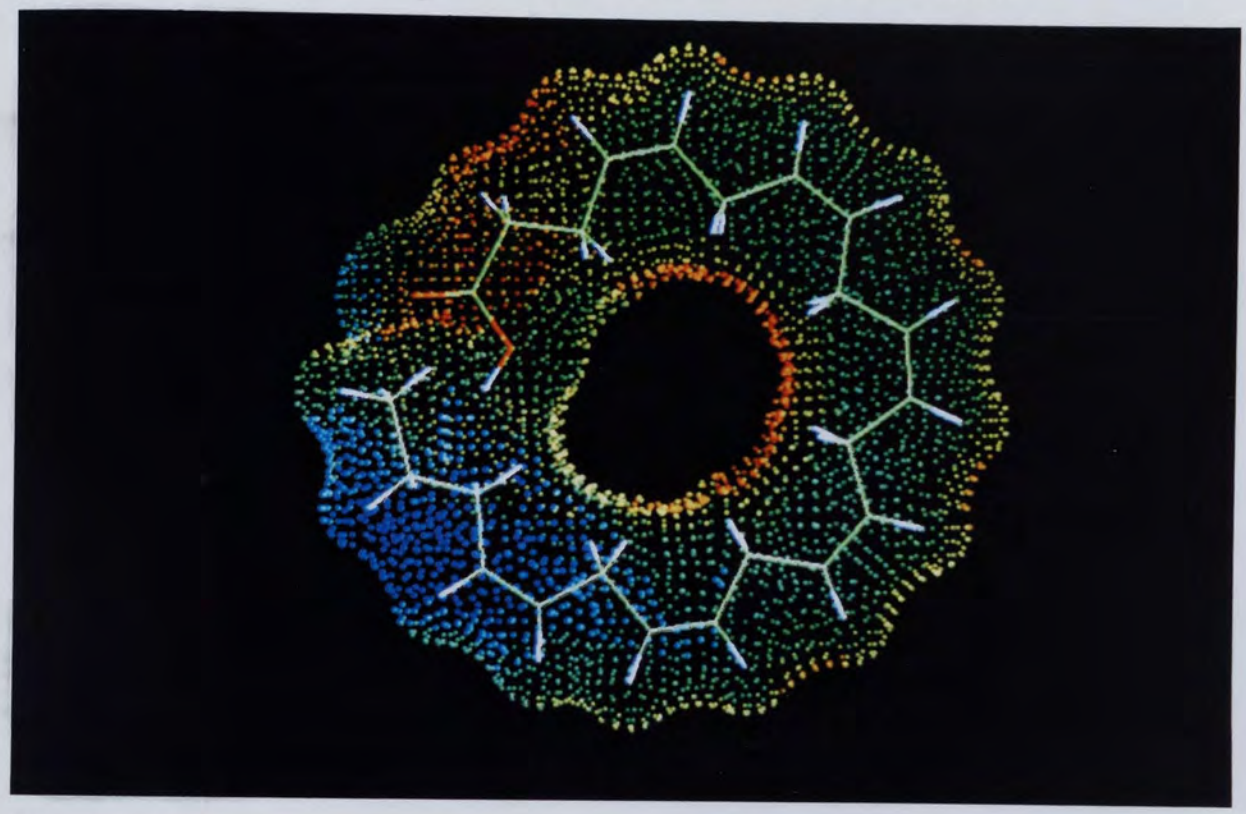


Figure 43B.

A Chemx molecular model of docosahexaenoic acid.

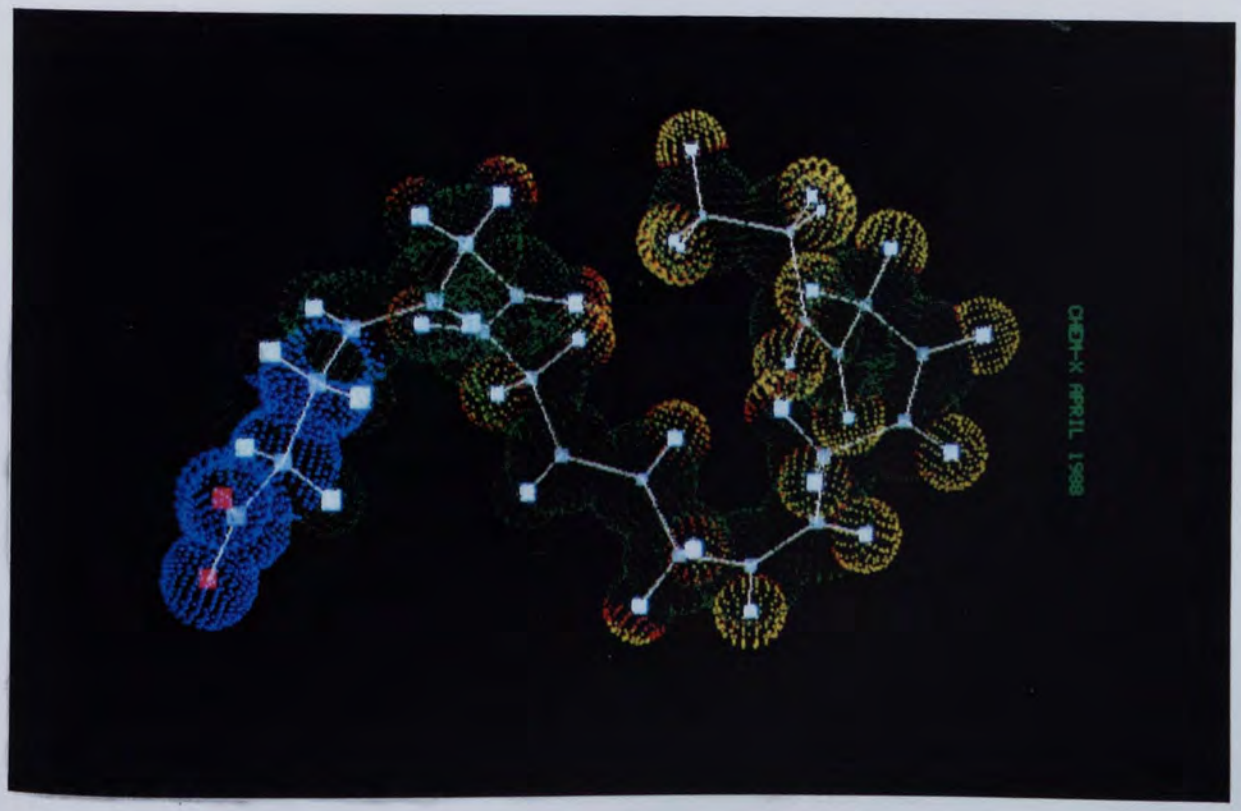


Figure 44A.

A Charmm molecular model of arachidonic acid.

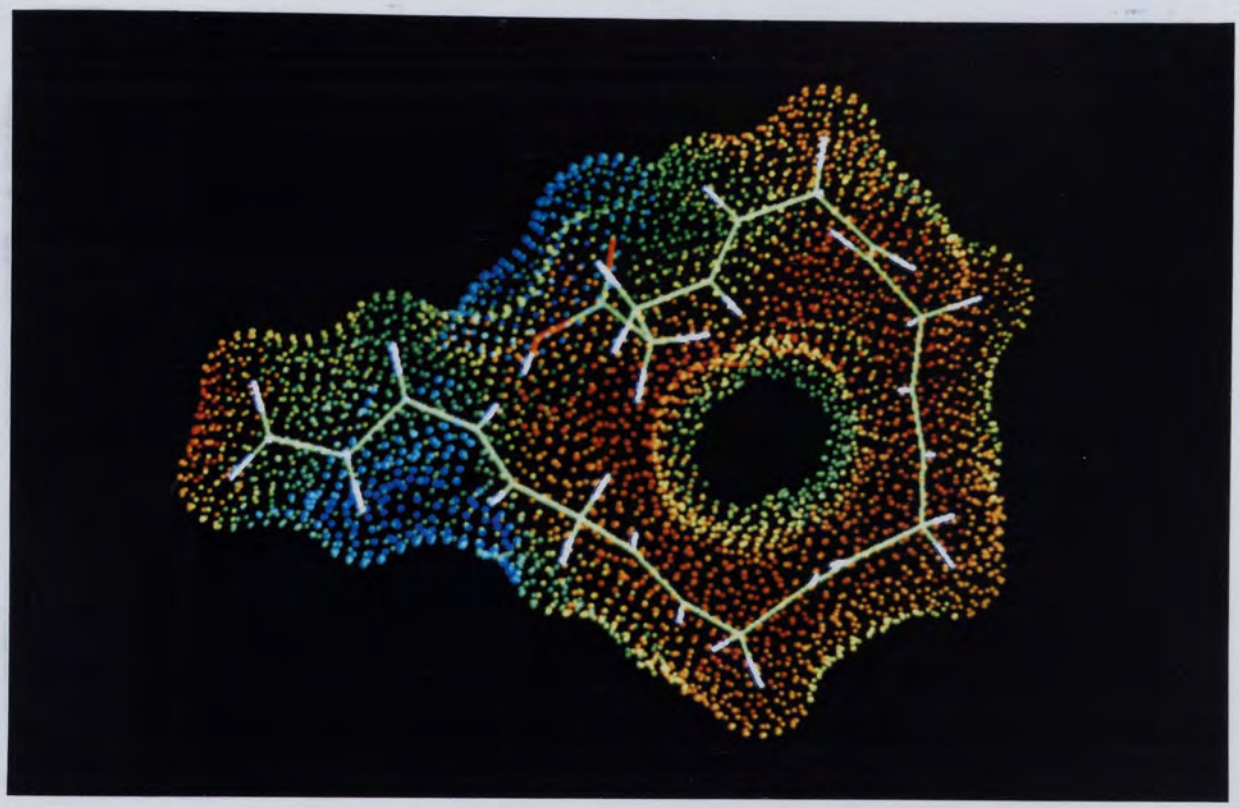


Figure 44B.

A Chemx molecular model of arachidonic acid.

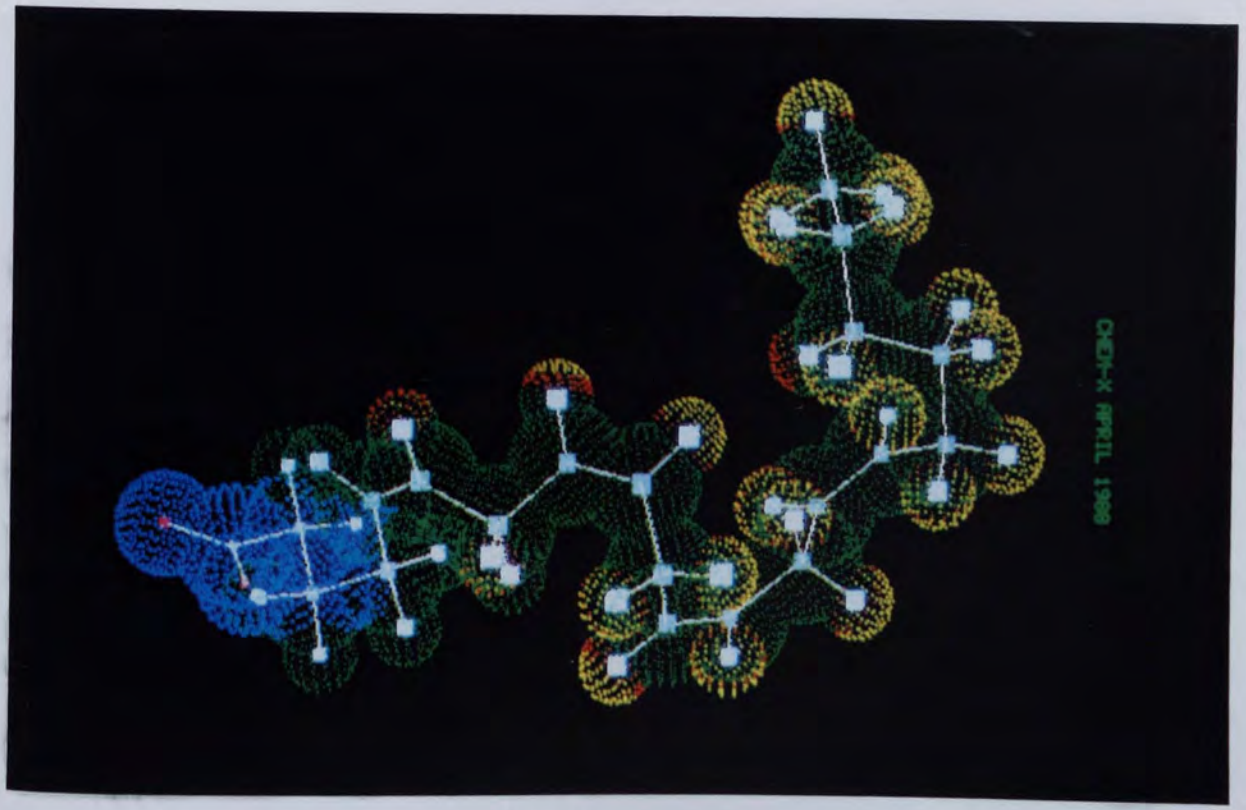


Figure 45A.

A Charmm molecular model of linolenic acid.

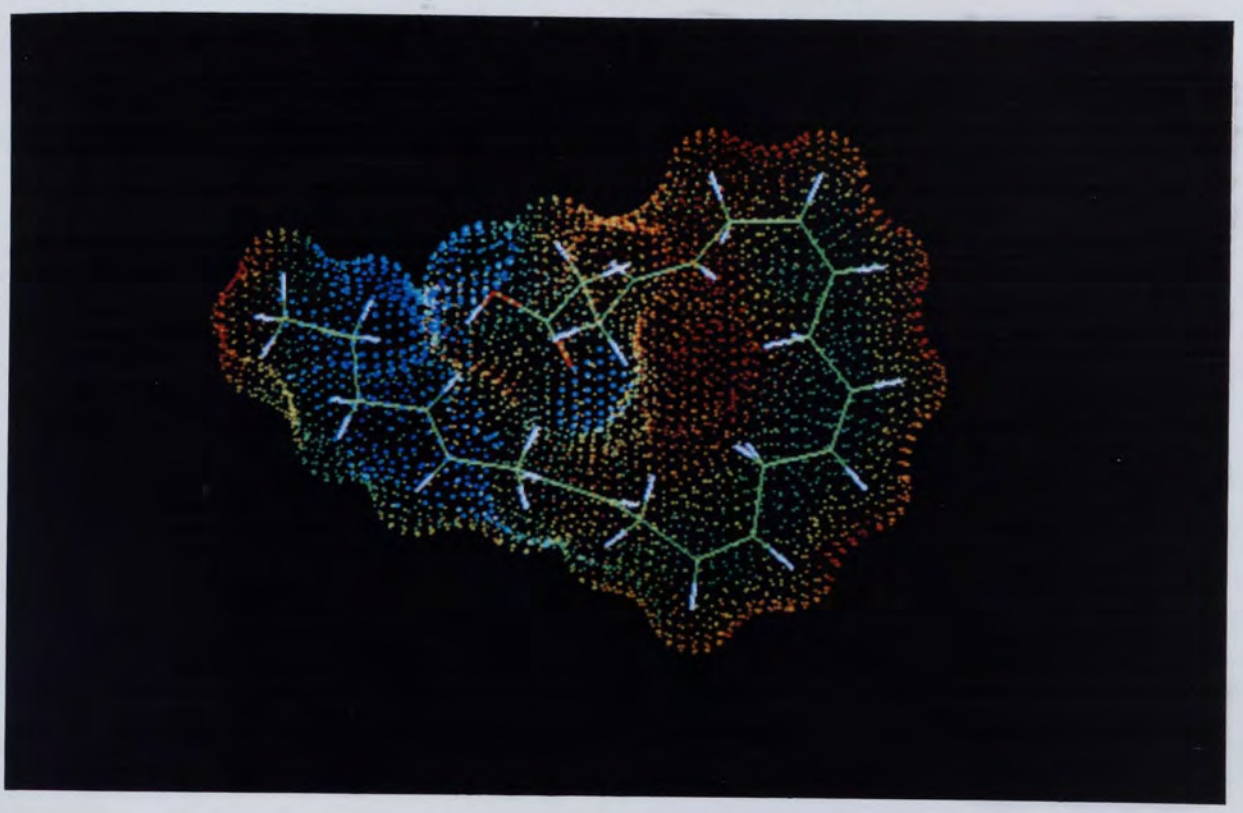


Figure 45B.

A Chemx molecular model of linolenic acid.

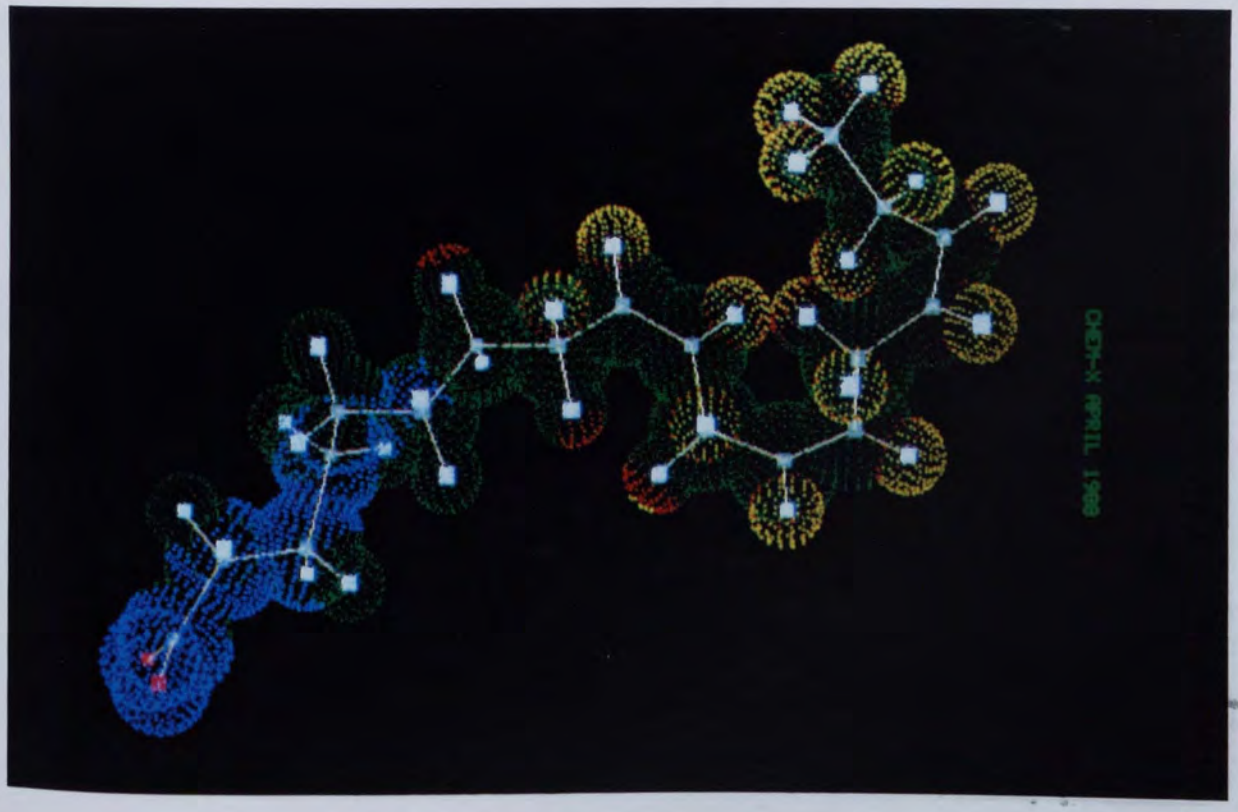


Figure 46A.

A Charmm molecular model of linoleic acid.

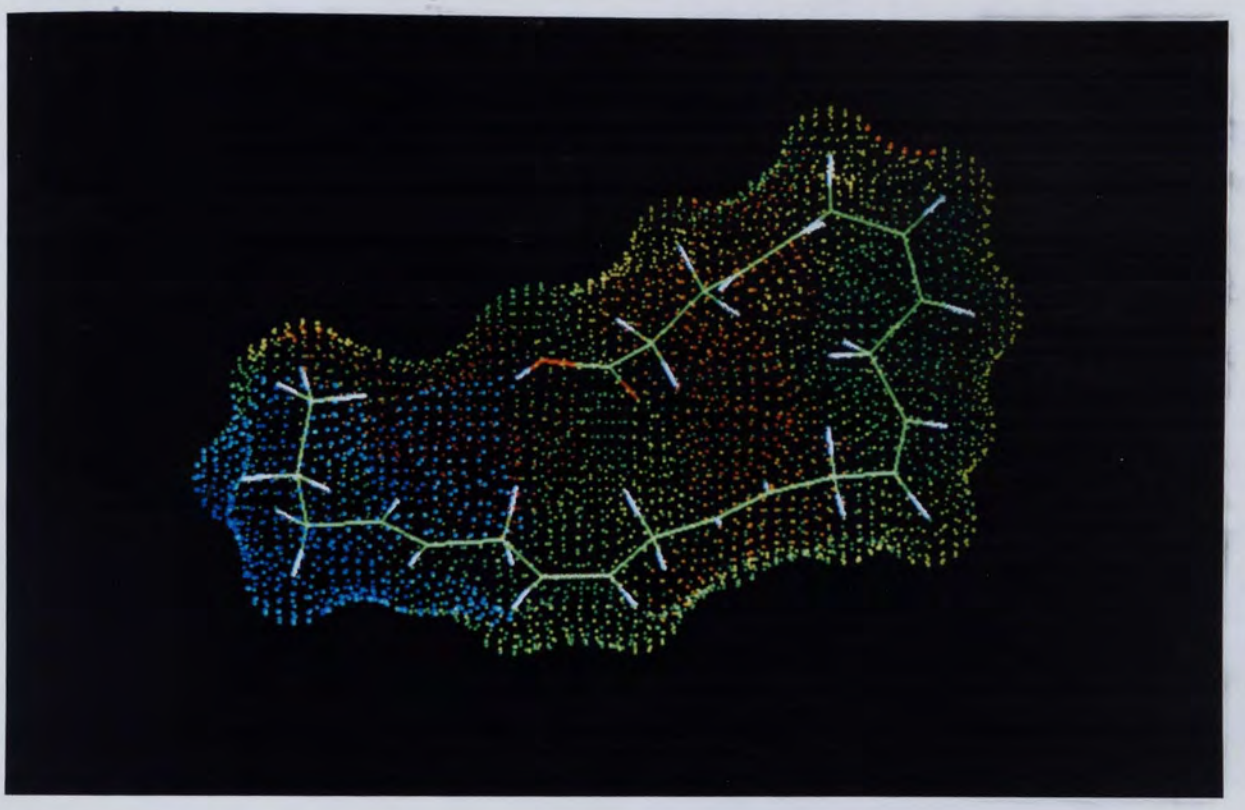


Figure 46B.

A Chemx molecular model of linoleic acid.

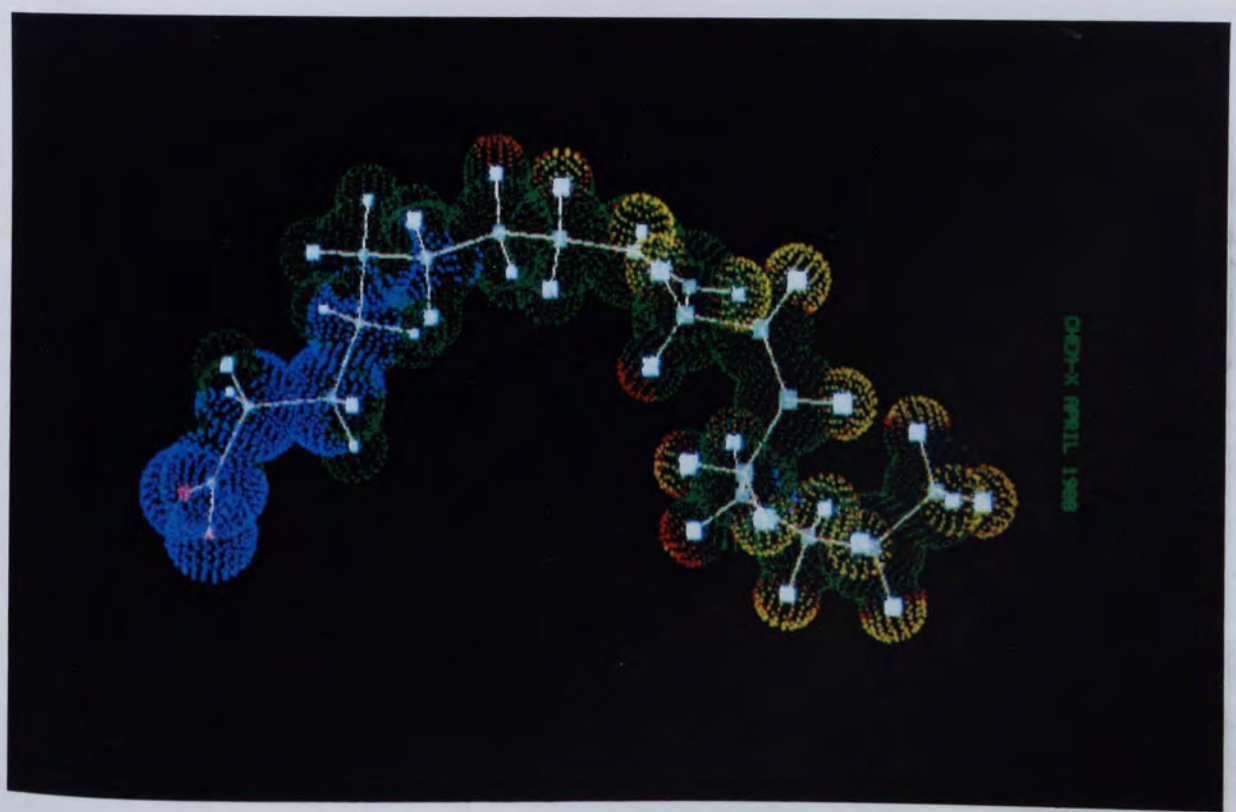


Figure 47A.

A Charmm molecular model of oleic acid.

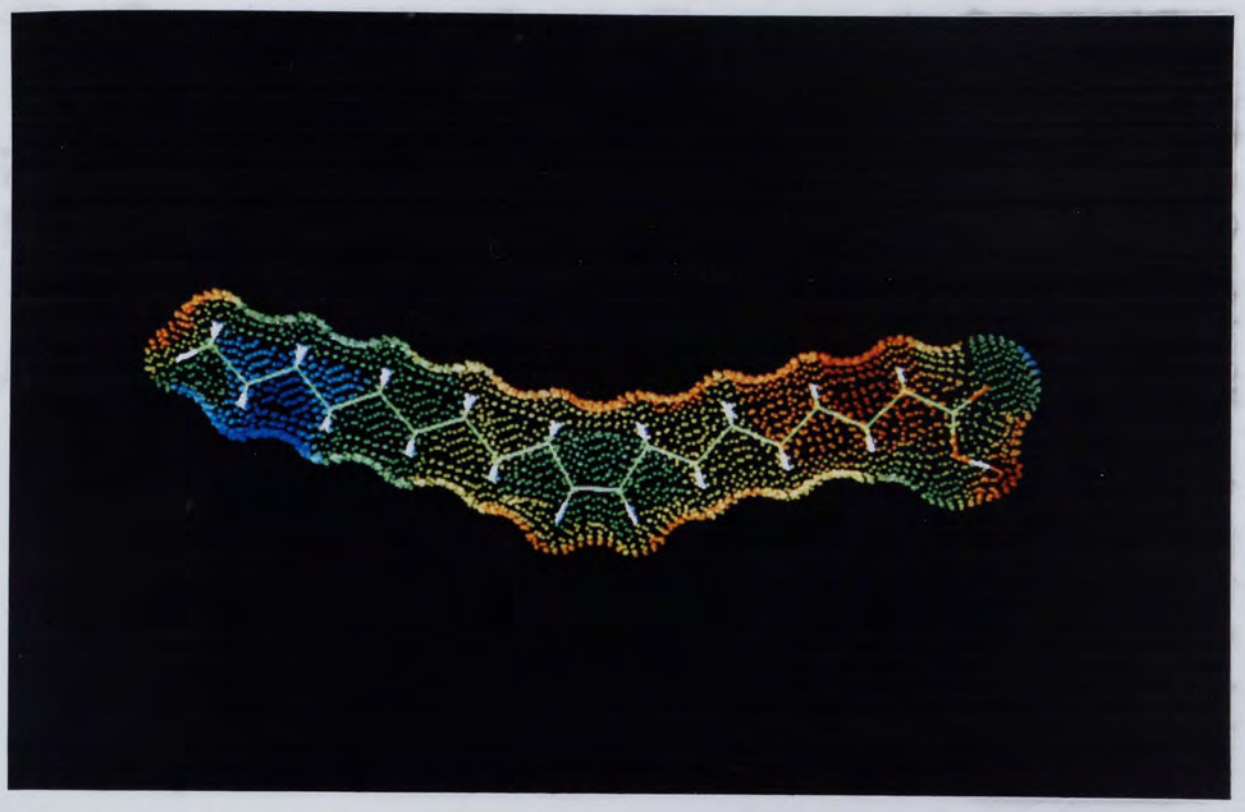
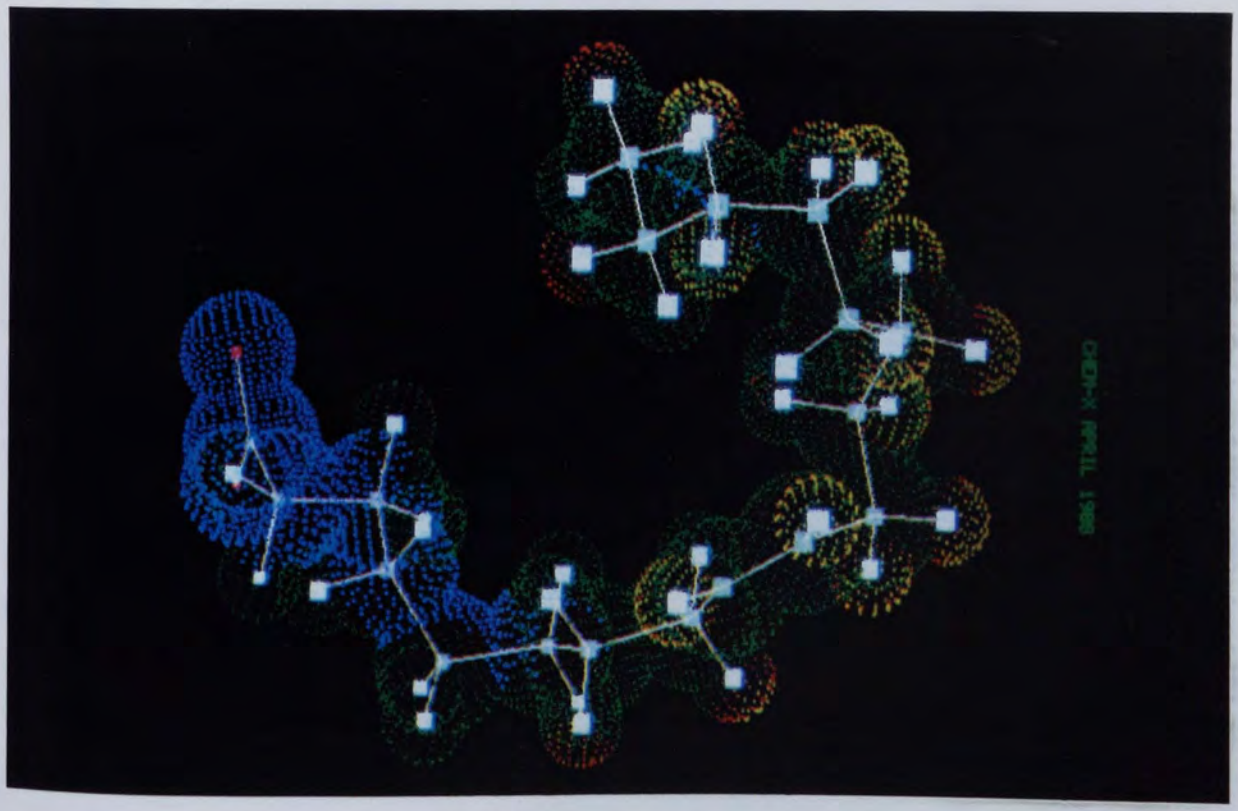


Figure 47B.

A Chemx molecular model of oleic acid.



correlation between any single variable, for example MW, or width of molecule, and enzyme activity. However, when four parameters were tried in combination (MW, length, width and number of double bonds) with the reciprocal of activity a near perfect correlation of R = 0.9816 was achieved - unfortunately the standard deviations and T-values were very large and inconclusive.

The regression analysis tries to "fit" the information into a linear/planar relationship in an attempt to correlate parameters to activity - in many biological systems such interactions do not have a linear relationship but a parabolic one. The computerised model of regression analysis did not have the capability to derive a parabolic relationship from the information supplied.

4.5.2. Discussion.

The Chemx and Charmm systems of molecular modelling are based largely on information derived from nuclear magnetic resonance studies. The major difference between the two systems is that Charmm is primarily a molecular mechanics program using predictive information from large proteins. Chemx utilizes parameters from a large base of widely differing small molecules and thus would appear to be better equipped to evaluate the structures of the fatty acids. In addition to achieve a set of coordinates that

represent the molecular conformation of the fatty acid with minimum potential energy the two systems also use differing minimization routines - these are discussed further in section 3.4.4. The models produced are theoretically midpoint between no structural information at all and the absolute structures elucidated using nuclear magnetic resonancy spectroscopy. It is probable that the Chemx models show a truer representation of the fatty acids than do the Charmm models - however they provide an interesting comparison.

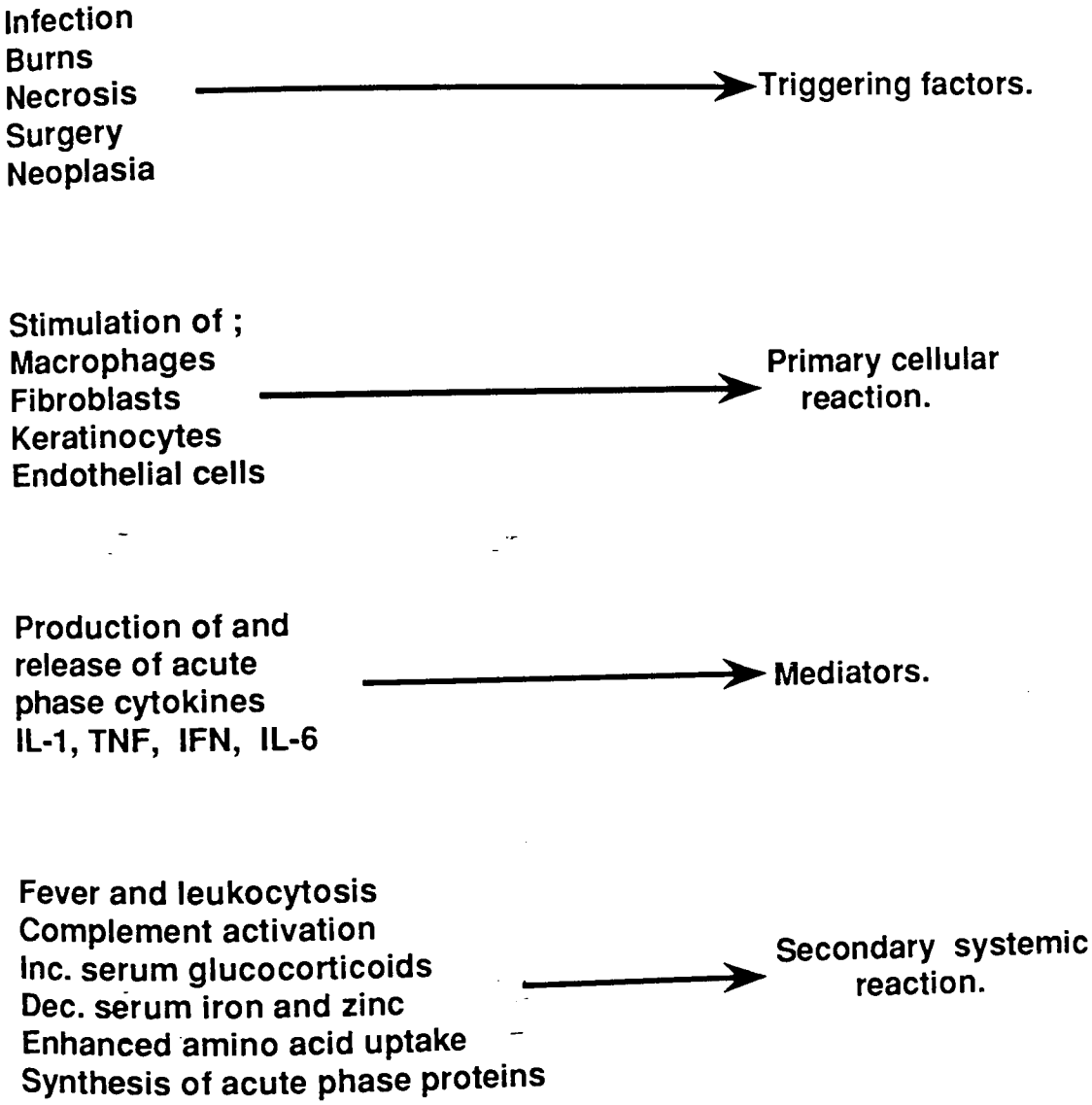
The conclusions that can be drawn from the regression analysis are limited - it is likely that the parameters identified otherwise interact in some as yet indefinable way to determine the degree of guanidinobenzoatase inhibition.

4.6. Characterisation of the potential circulating mediator of muscle proteolysis associated with MAC16 colon adenocarcinoma induced cachexia.

4.6.1. Introduction.

The tumour may also directly or indirectly influence host metabolism to bring about the cachectic state via the acute phase response. A summary of the acute phase response is shown in fig.48. The metabolic consequences of the acute

Figure 48.



A summary of the acute phase response.

(Roitt et al, 1988)

phase response include the development of fever, anorexia, changes in carbohydrate, protein and fat metabolism and alterations in the concentration of a large number of plasma proteins - reflecting the reorchestration of the pattern of gene expression and secretory proteins in hepatocytes. All of these reactions are probably beneficial to the host, at least in the short term, mobilising host defense mechanisms against the inciting disease process. If the disease initiating the acute phase response is eradicated, the stage of resolution and healing begins. However, if the host is unable to rid itself of the injurious agent, a chronic state may ensue and the adaptive homeostatic reactions that were initially beneficial may ultimately be detrimental.

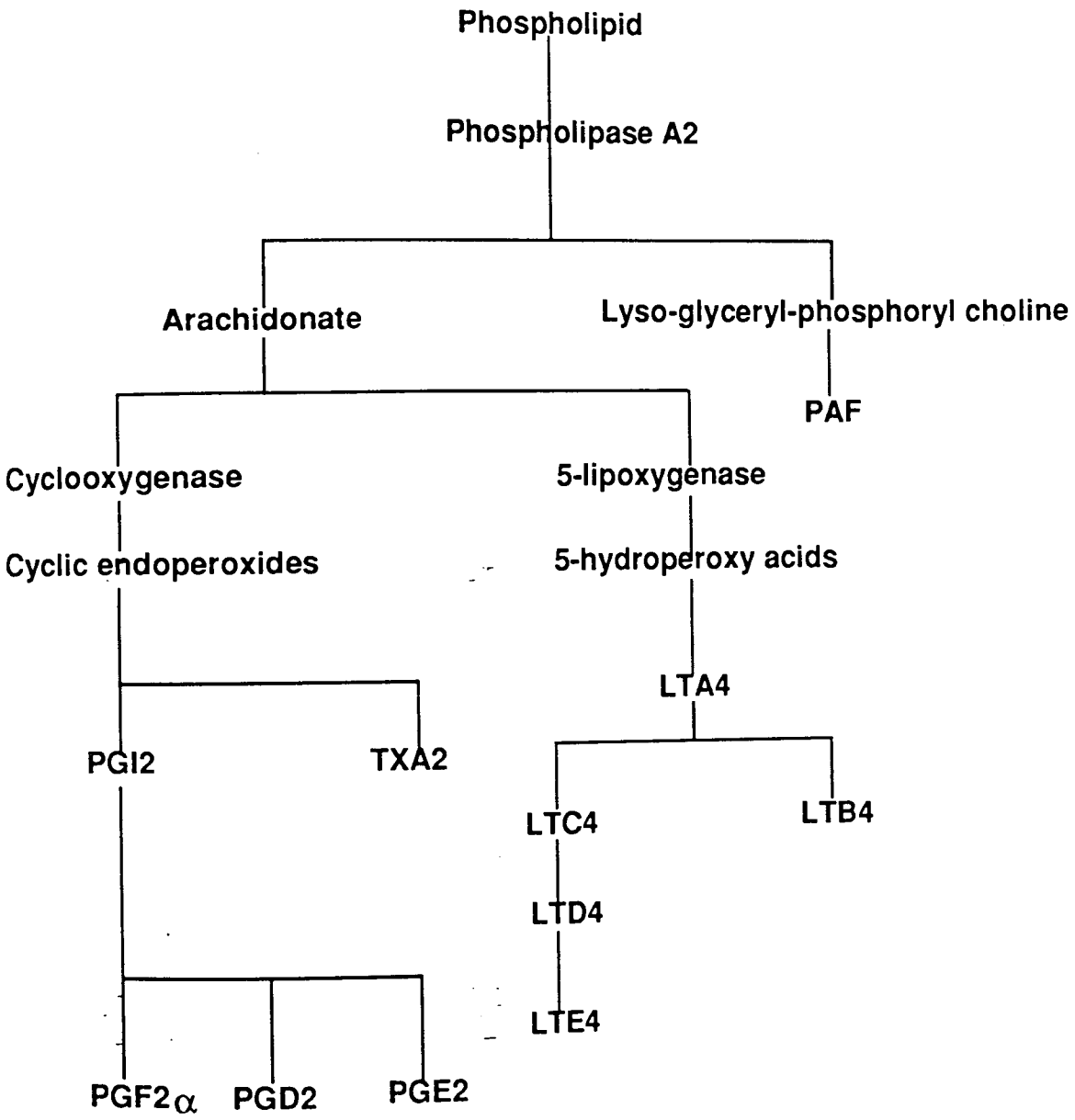
Clowes et al (1983) have identified a circulating peptide called proteolysis-inducing factor (PIF) in patients with sepsis or trauma. This hormone-like protein secreted by activated monocytes may be IL-1 or IL-1-like (Baracos et al, 1983). Similarly Belizario et al (1991) found a proteolysis-inducing factor in the plasma proteins of 25 out of 50 cancer patients with weight loss and in 5 of these samples the bioactivity was partially abrogated with antibodies to recombinant human IL-1. Thus the accelerated breakdown of proteins by the cancer plasma factors was in this case partially mediated by IL-1 in cooperation with additional undefined factors. However, Moldawer et al

(1987) refute this view since recombinant IL-1 proteins do not stimulate mouse or rat muscle catabolism in vitro.

The tumour may also mediate muscle proteolysis through a family of substances called the eicosanoids. They are generated denovo from phospholipids in response to a surprisingly wide range of different stimuli and their presence has been detected in virtually every tissue in the body. They are implicated in the control of many physiological processes and they are among the most important mediators and modulators of the inflammatory reaction (Rang and Dale, 1988). A summary diagram of the mediators derived from phospholipids is shown in Fig.49.

In addition to their role in inflammation the prostaglandins are thought to play a controversial role in the regulation of protein synthesis and degradation in particular in sepsis and trauma. Rodemann et al (1982) observed that protein degradation was increased by arachidonic acid in both the tonic soleus and in the phasic extensor digitorum longus, diaphragm and heart muscles of rats in vitro and that aspirin and indomethacin inhibited the effect. Prostaglandin E_2 was similarly effective in stimulating protein degradation but prostaglandin $F_{2\alpha}$ was not. Protein synthesis was also stimulated by arachidonic acid but only in the soleus muscle and prostaglandin $F_{2\alpha}$ mimicked this effect. These observations gave rise to the

Figure 49.



PG = Prostaglandin, PGI2 = Prostacyclin, TX = Thromboxane,
LT = Leukotriene, PAF = Platelet activating factor.

A summary of the mediators derived from phospholipids.

(Rang and Dale, 1988)

hypothesis that PGE_2 and $PGF_{2\alpha}$ regulated muscle turnover by their effects on protein synthesis and degradation. In addition inhibitors of lysosomal thiol proteases inhibited the stimulation of protein degradation by arachidonic acid and PGE_2. Consequently intralysosomal proteolysis has been implicated as the mediator. However, other studies - Barnett and Ellis (1987) in particular, fail to substantiate this view. Palmer and Wahle (1987) were unable to demonstrate in vitro a significant stimulation of protein degradation in rabbit forelimb digit extensor muscles with the addition of arachidonic acid but a stimulation of protein synthesis was evident. Freund et al (1985) could not determine any significant difference in the prostaglandin production from endogenous precursors between control animals with moderate injury and septic animals with severe injury. The relationship between cancer and prostaglandins is a complex one. Human tumours and experimentally induced animal tumours have been shown to produce prostaglandins - for example colonic, breast, thyroid and renal cell carcinomas, lymphoma, neuroblastoma, pheocromocytoma, islet cell tumour and mouse sarcoma and fibrosarcoma (Husby et al, 1977, Levine, 1981). The pathophysiological result of this prostaglandin production manifests itself as immunosupression (Roitt et al, 1987), tumour cell migration (Young and Newby, 1986) and bone resorption (Tashijian et al, 1974). The possible role of

prostaglandins in the mediation of muscle catabolism in sepsis and trauma may also be a potential effector mechanism in the muscle wasting associated with cancer cachexia. Strelkov et al (1989) investigated the effect of naproxen,(a cyclooxygenase inhibitor that binds reversibly to the enzyme competing with the natural substrate arachidonic acid) on two experimental tumour models that cause severe wasting and muscle catabolism - the Morris hepatoma and the Yoshida ascites hepatoma AH130. The major difference between these two models is that the Yoshida hepatoma is known to produce prostaglandins. Naproxen treatment inhibited prostaglandin production in the Yoshida ascites hepatoma and induced muscle atrophy and protein loss was inhibited by 40%. However, in the Morris hepatoma naproxen treatment had no effect. Thus it would appear that the muscle loss seen in cachexia, in addition to the numerous other mechanisms mentioned, may be partially mediated by either a prostaglandin dependent or independent mode of action depending on tumour type.

The hosts immunological response in addition to part of the innate immune system already mentioned may also be subverted to the tumours benefit - an example of this would be immunological escape (Roitt et al, 1988). A situation can also be envisaged where the tumour sheds antigens that mimic the "self" receptor molecules of skeletal muscle that control protein turnover, an immune response is elicited

and a cascade of events is persistently initiated until a down regulation of effector molecules is achieved. However, this is purely speculative.

The aim of this section is to identify and characterise the circulating proteolytic factor.

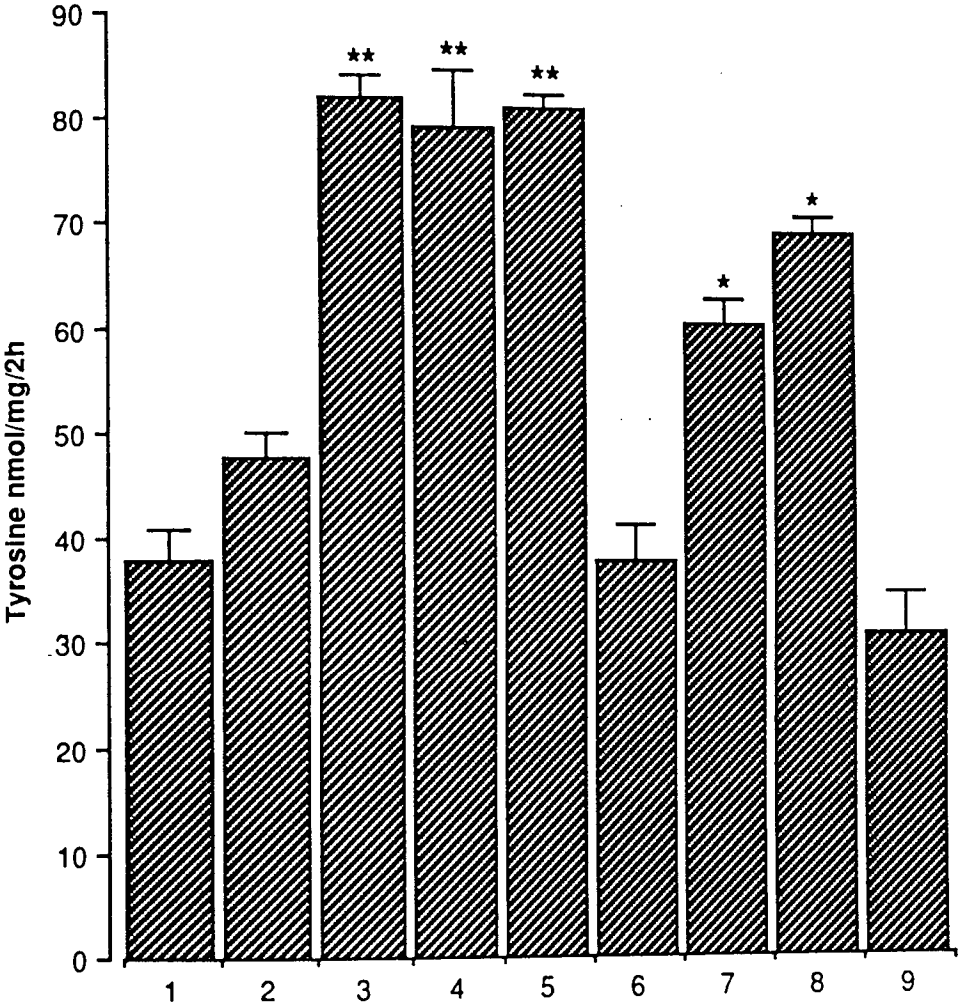
4.6.2. Results.

The proteolytic activity of serum from non tumour-bearing animals, MAC16 tumour-bearing animals with weight loss and MAC13 tumour-bearing animals was compared (fig.50). Serum from animals bearing the MAC16 tumour with weight loss contained significantly higher levels of the protein degradative factor than the MAC13 tumour-bearing animals or the non tumour-bearing animals.

The nature of the proteolytic factor was investigated using various treatments that by their action, would exclude certain elements from the serum of cachectic animals. The protein degradative factor was significantly inhibited by indomethacin (a cyclooxygenase inhibitor,200μM), EPA (a PGE_2 synthesis inhibitor, 500μM) and BW4AC (a lipoxygenase inhibitor, 1.77mM). Heating the serum to 60°C for 5 min and treatment with phenylmethylsulphonyl fluoride (a serine protease inhibitor, 1mM) failed to reduce the degradative effect of the factor. The effect of the indomethacin

Figure 50.

The effect of various treatments on tyrosine release from isolated gastrocnemius muscle.



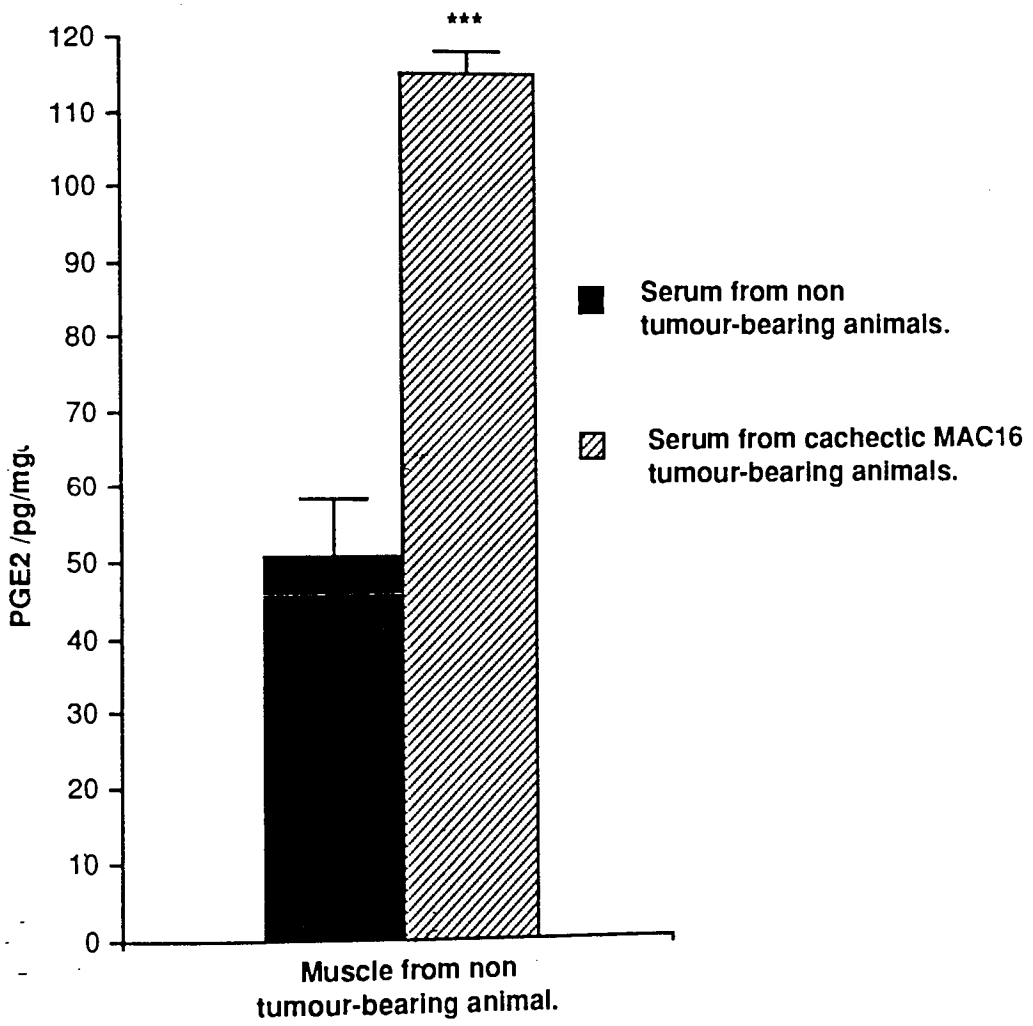
1. Serum from non tumour-bearing animals.
2. Serum from MAC13 tumour-bearing animals.
3. Serum from cachectic MAC16 tumour-bearing animals.
4. Serum from 3. + 1mM PMSF.
5. Serum from 3. + 1mM PMSF.
6. Serum from + 200μM indomethacin.
7. Serum from 3. + 500μM EPA.
8. Serum from 3. + 1.77mM BW4AC.
9. Partially purified MAC16 lipolytic factor.

Each bar represents the mean ± SEM of 3 animals.
Differences were determined by one-way ANOVAR as *= $P<0.05$, **=$P<0.01$ compared to group 1.

suggested that the increase in protein degradation seen with serum from MAC16 tumour-bearing animals with weight loss may be attributable to PGE_2. Consequently the PGE_2 content of muscles from non tumour-bearing animals was measured after a 2h incubation with either sera from non tumour-bearing animals or animals bearing the MAC16 tumour with weight loss (fig.51). This suggests that some component of serum from MAC16 tumour-bearing animals with weight loss induces PGE_2 formation in muscle. Indomethacin significantly reduced the PGE_2 content of isolated gastrocnemius muscle in a dose dependent manner and this occured concomitantly with a reduction in tyrosine release (fig.52). However, a 66% decrease in the PGE_2 content of the muscle was required before there was a significant reduction in the activity of the proteolytic factor. It was proposed that the component in serum from cachectic animals that increases the PGE_2 content of muscles may be a prostaglandin precursor - for example arachidonic or linoleic acid as the serum level of arachidonate is significantly increased in cachectic animals. However, the addition of these PUFAs to isolated gastrocnemius muscle preparations either in free solution or bound to non tumour-bearing mouse serum did not increase protein degradation to a level comparable to that seen with serum from cachectic MAC16 tumour-bearing animals. The PUFA EPA is an effective inhibitor of weight loss in MAC16 tumour-bearing animals. The maintenance of skeletal muscle

Figure 51.

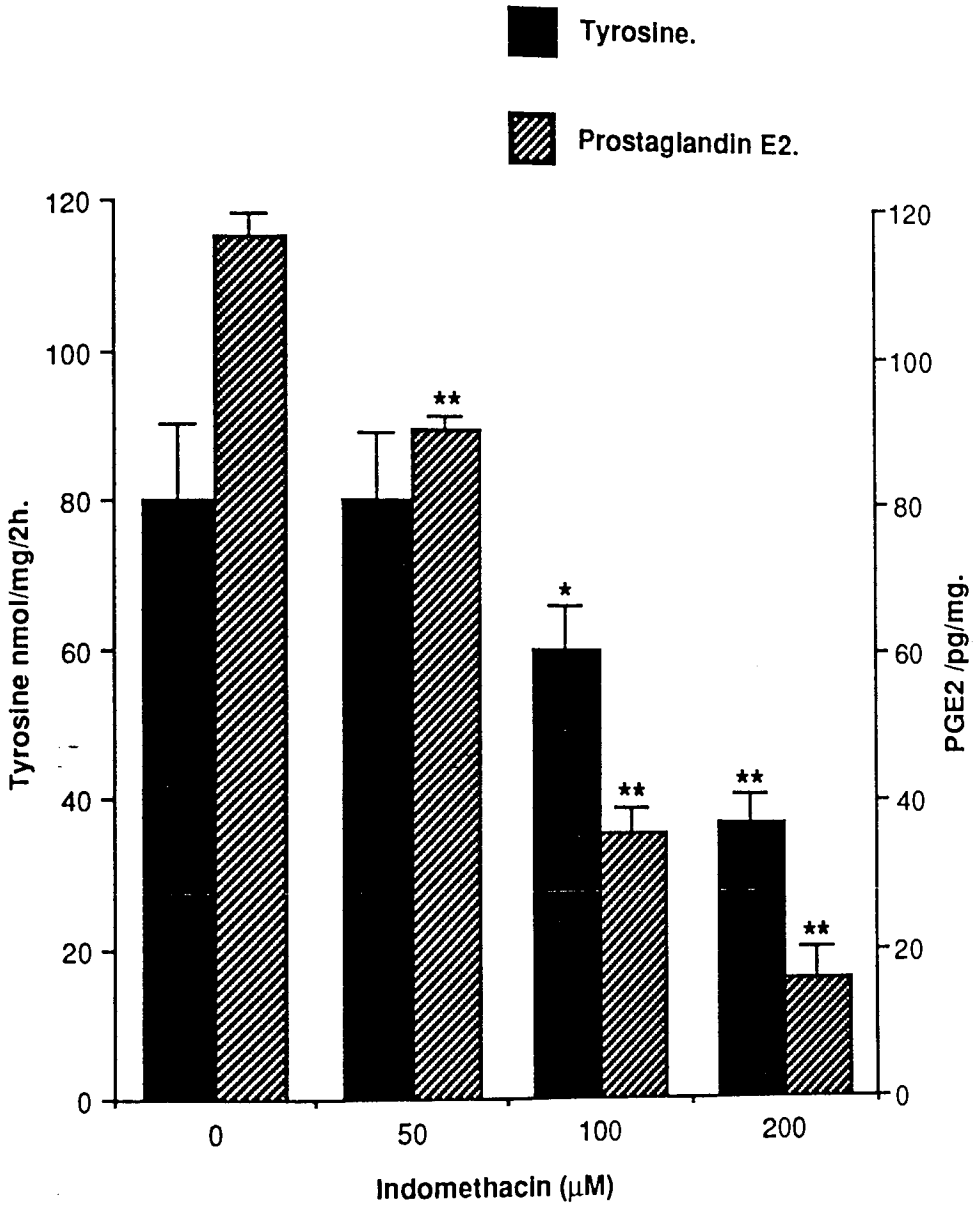
The effect of serum from non tumour-bearing animals and MAC16 tumour-bearing animals with 11-15% weight loss on isolated gastrocnemius muscle PGE2 content.



Each bar represents the mean ± SEM of 6 animals. Differences were determined by Students T-test as *** =P<0.001 from muscles treated with serum from non tumour-bearing animals.

Figure 52.

The effect of indomethacin on the PGE2 content of and tyrosine release from isolated gastrocnemius muscle.

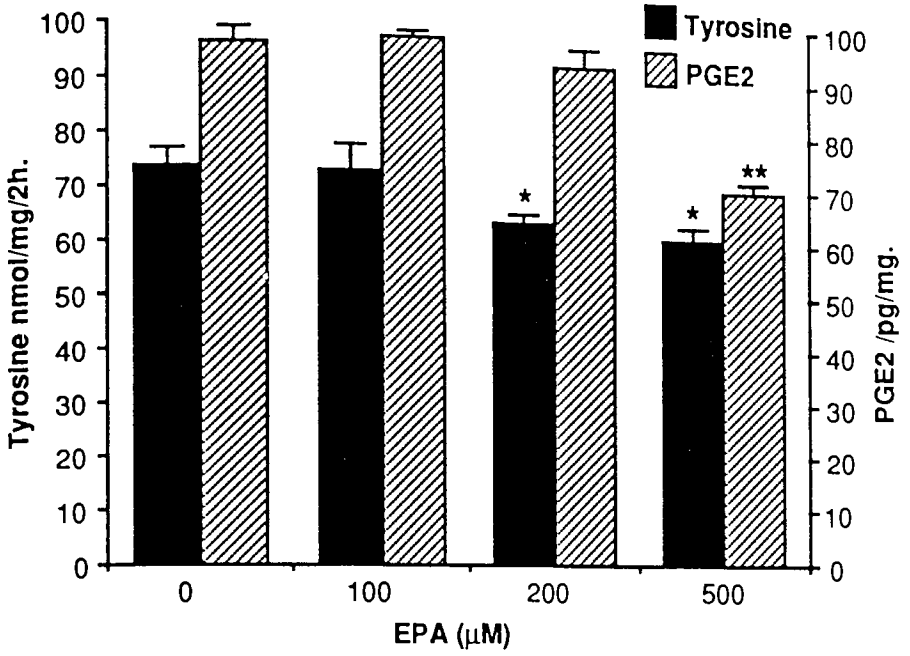


Each bar represents the mean ± SEM for 12 determinations per group
Differences were determined by one-way ANOVAR as *=P<0.05,
**=P<0.01 compared to muscles incubated in the absence of indomethacin.

in such animals may be attributable to a 60% reduction in muscle protein degradation (section 4.0.2.). The results in figs.53A and 53B compare the effect of EPA and DHA on the PGE_2 content of, and tyrosine release from isolated gastrocnemius muscle under the influence of serum from cachectic MAC16 tumour-bearing animals. DHA has no effect whilst EPA inhibits tyrosine release and PGE_2 content by 18% and 27% respectively. In fig.54 the effect of serum from non tumour-bearing animals and MAC16 tumour-bearing animals with weight loss is seen on muscles from non tumour-bearing animals and MAC16 tumour-bearing animals previously treated with EPA 2.0g/kg-1 for 5 days. The serum from cachectic animals caused a significant increase in tyrosine release from muscles from non tumour-bearing animals when compared to serum from non tumour-bearing animals. However, in gastrocnemius muscles isolated from animals previously treated with EPA the effect of the serum from cachectic animals was considerably moderated to a level that was not significantly different from that produced by serum from non tumour-bearing animals. This experiment was repeated using only serum from MAC16 tumour-bearing animals with weight loss - muscle PGE_2 content was also measured (fig.55). EPA pre-treatment diminished the degradative effect of serum from cachectic animals by 58%. The PGE_2 content of the EPA pre-treated gastrocnemius muscle was similarly reduced by 49%. In figs.56A and 56B the effect of the triglycerides -

Figure 53A.

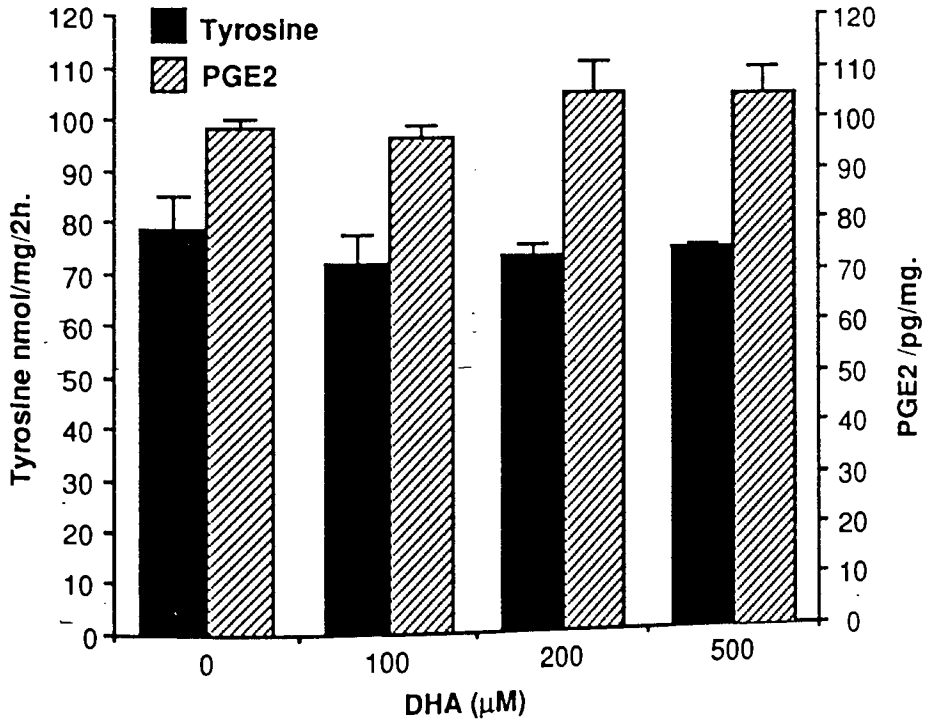
The effect of EPA on the PGE2 content of and tyrosine release from isolated gastrocnemius muscle.



Each bar represents the mean ± SEM of 3 animals. Differences were determined by one-way ANOVAR as *= P<0.05, **= P<0.01 compared to the control (0).

Figure 53B.

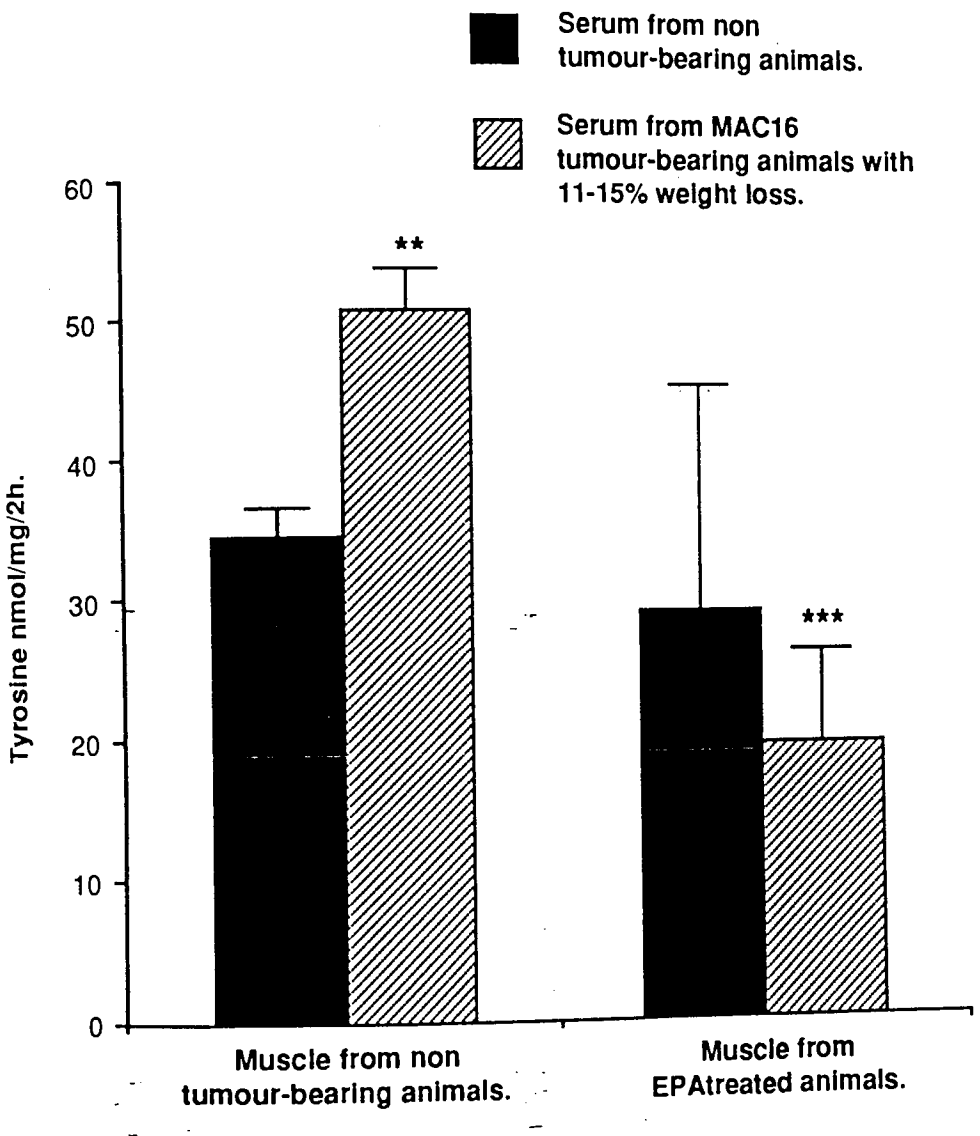
The effect of DHA on the PGE2 content of and tyrosine release from isolated gastrocnemius muscle.



Each bar represents the mean ± SEM of 3 animals. There were no significant differences between the above groups.

Figure 54.

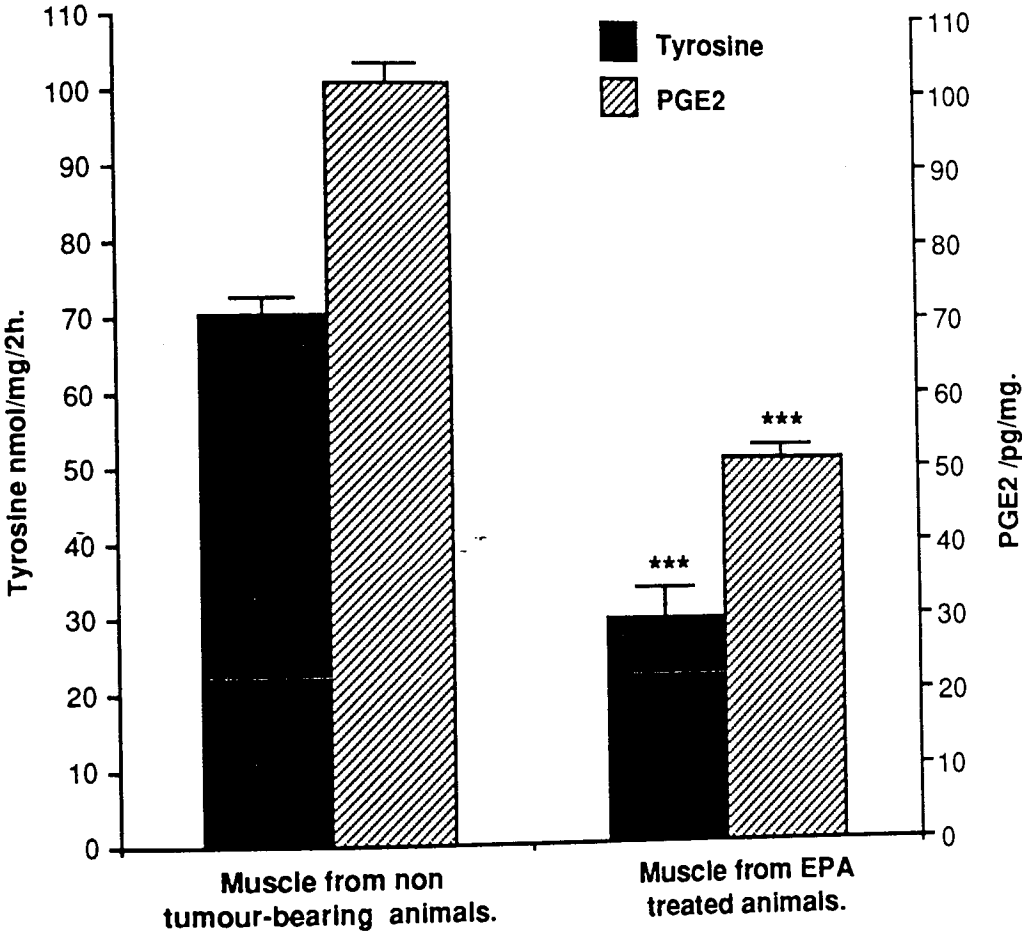
The effect of treatment of animals with EPA on the response of isolated gastrocnemius muscle to the induction of protein degradation by serum.



Each bar represents the mean ± SEM of 4 animals. Differences were determined by one-way ANOVAR as **=P<0.01 from tyrosine released in the presence of serum from non tumour-bearing animals and ***=P<0.001 from degradation observed in muscle from non tumour-bearing animals.

Figure 55.

The effect of treatment of animals with EPA on the PGE2 content of and tyrosine release from isolated gastrocnemius muscle in response to serum from cachectic MAC16 tumour-bearing animals.



Each bar represents the mean ± SEM of 3 animals.
Differences were determined by Students T-test as *** = P<0.001 from muscles from non tumour-bearing animals.

Figure 56A.

The effect of serum from non tumour-bearing mice supplemented with triarachidonin on tyrosine release from isolated gastrocnemius muscle.

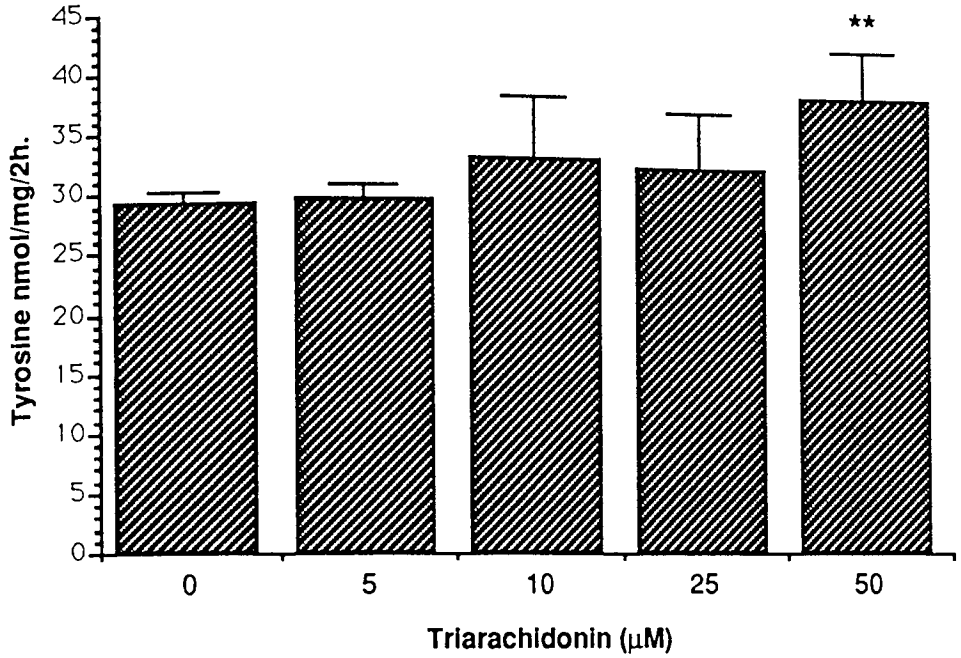
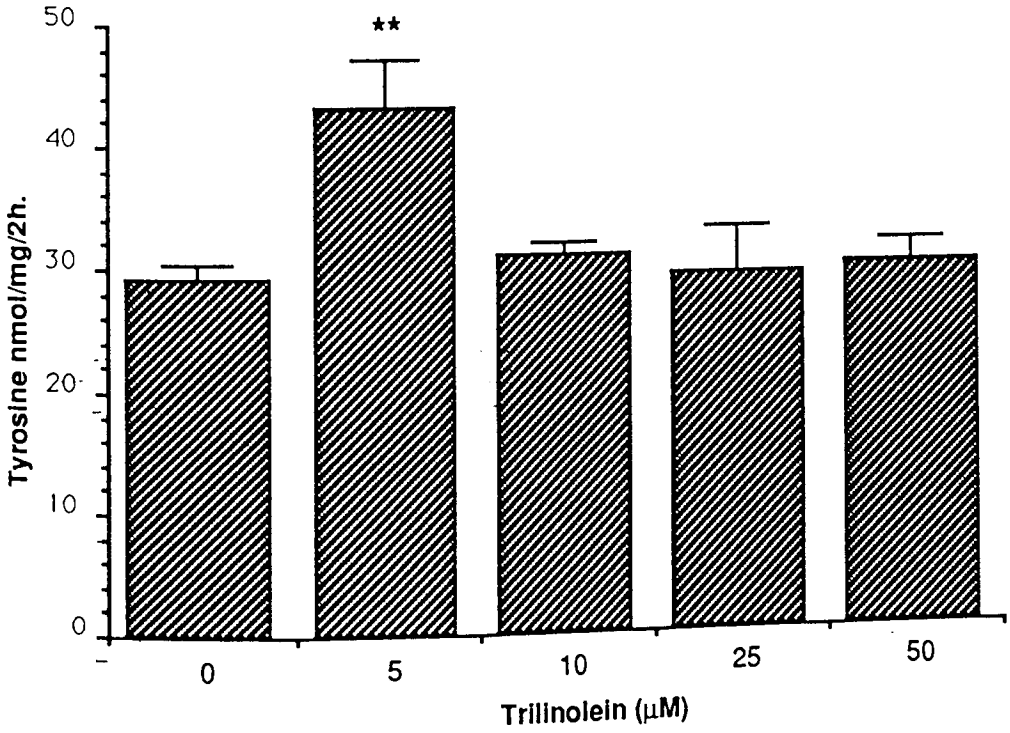


Figure 56B.

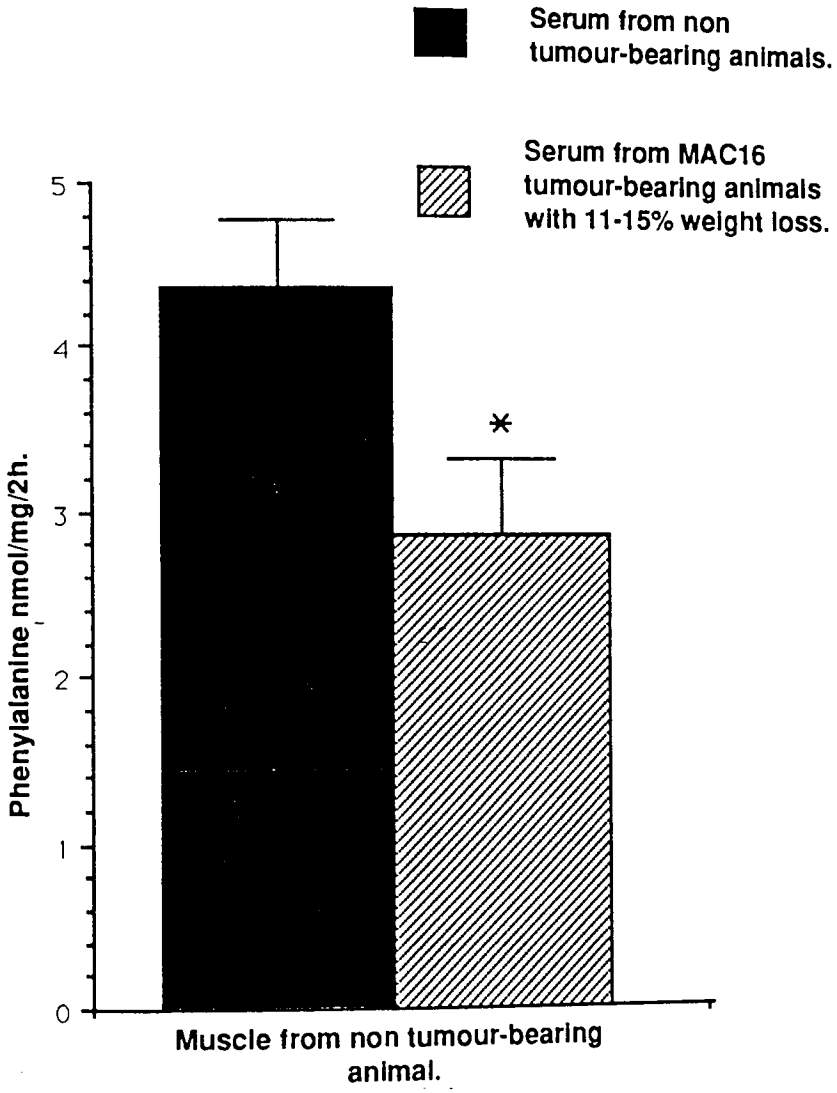
The effect of serum from non tumour-bearing mice supplemented with trilinolein on tyrosine release from isolated gastrocnemius muscle.



Each bar represents the mean ± SEM for 3 animals. Differences were determined by one-way ANOVAR as **=P<0.01 from the control group.

Figure 57.

The effect of serum from non tumour-bearing and cachectic MAC16 tumour-bearing animals on isolated gastrocnemius muscle protein synthesis.



Each bar represents the mean ±SEM for 3 animals. Differences were determined by Student's T-test as*=P<0.05 from muscle treated with non tumour-bearing mouse serum.

triarachidonin and trilinolein added to serum from non tumour-bearing animals is shown on the extent of tyrosine release from isolated gastrocnemius muscle. At concencentrations of 50μM and 5μM triarachidonin and trilinoein stimulated protein degradation by 33% and 48% respectively. The results presented in fig.57 show the effect of serum from non tumour-bearing and MAC16 tumour-bearing animals with weight loss on gastrocnemius muscle protein synthesis. Serum from cachectic animals causes a 35% decrease in protein synthesis when compared with serum from non tumour-bearing animals.

4.6.3. Discussion.

The nature of the MAC16 proteolytic factor is not yet fully known - however, preliminary findings would suggest that it is heat stable, it is not a serine protease and its action is not mediated directly by the MAC16 lipolytic factor that is currently under investigation. The factor is not produced as a consequence of tumour-bearing alone, as serum from MAC13 tumour-bearing animals is comparable to serum from non tumour-bearing animals in its ability to elicit tyrosine release from isolated gastrocnemius muscle. Indomethacin a cyclooxygenase inhibitor completely abolishes the proteolytic action of serum from cachectic animals to base levels. High concentrations of EPA and BW4AC also significantly compromise activity - although the

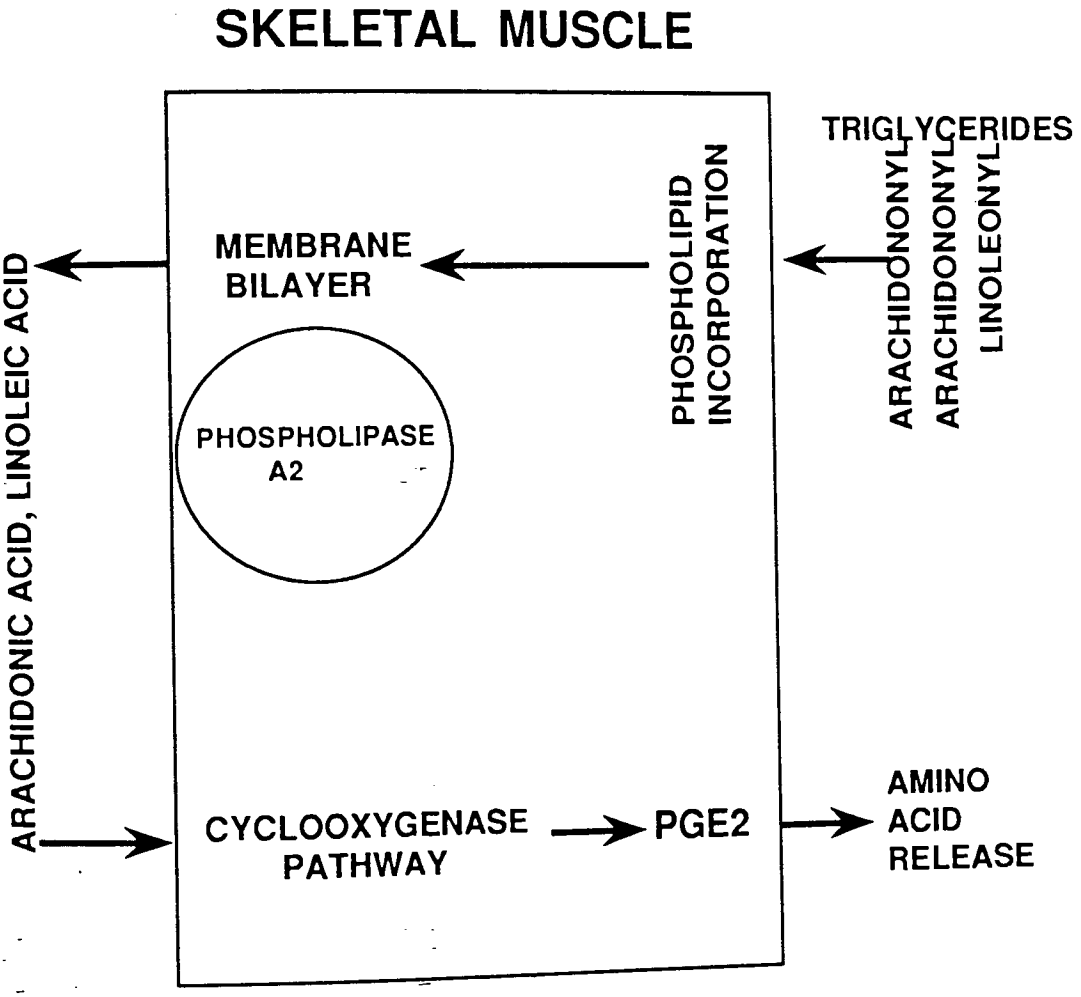
concentration of BW4AC that was used far exceeds its IC50 value for the inhibition of lipoxygenase (Tateson et al, 1988) and may even be toxic. This suggests that the effect may be mediated through a prostaglandin intermediate. Indomethacin in particular and also EPA inhibits the PGE_2 content of muscle in a dose dependent manner in response to serum from cachectic animals. This would suggest that the proteolytic factor mediates its effect largely through the cyclooxygenase/prostaglandin pathway rather than through the lipoxygenase/leukotriene pathway. The role of prostaglandins, in particular PGE_2, is demonstrated by the fact that gastrocnemius muscle contains approximately 50% more PGE_2 under the influence of serum from cachectic animals than serum from non tumour-bearing animals. EPA not only reduces the proteolytic capacity of serum from cachectic animals, but as a prostaglandin synthesis inhibitor (Levine and Worth, 1984) also reduces the PGE_2 content of the gastrocnemius muscle in vitro and in vivo. DHA has no inhibitory effect - this is possibly because in vitro the fatty acid does not become sufficiently incorporated in the muscles cellular lipids where it can compete with aracidonic acid for the cyclooxyenase. EPA in vivo has been shown to moderate protein loss in the MAC16 tumour bearing animal through a reduction in protein degradation (section 4.0.2.) - gastrocnemius muscle from these animals is more resistant to the degradative effect of serum from cachectic animals than gastrocnemius muscle

from non tumour-bearing animals. This would suggest that EPA influences protein degradation by inhibiting the proteolytic factor thus reducing the PGE_2 content of the muscle by inhibiting PGE_2 synthesis and thus preventing further degradation by serum from cachectic animals.

Since the level of arachidonic acid in the serum of cachectic animals is significantly elevated (Hudson, 1992, unpublished results) the possibility that arachidonic acid (as a prostanoid precursor) was the mediator of the increased proteolysis in cachectic animals was investigated. Contrary to the findings of Rodeman and Goldberg (1982) no stimulation of protein degradation was achieved with either arachidonic on linoleic acid. However, arachidonic and linoleic acid are not normally found in serum in the free fatty acid form, they usually exist as mixed triglycerides - thus the effect of triarachidonin and trilinolein was investigated. The cummulative effect of these triglycerides was an 81% increase in protein degradation. It would appear that a high ratio of triarachidonin to trilinolein would be required in vivo to produce the effect of increased degradation - however, in reality, as mentioned earlier these synthetic triglycerides do not exist and it would be more likely that it is the fatty acid composition of natural triglycerides that may be responsible for the degradative effect of serum from cachectic MAC16

tumour-bearing animals. The concentration of fatty acids in serum hydrolysed from triglycerides, possibly under the influence of the MAC16 lipolytic factor, is currently under investigation. The proposed mechanism of action of the proteolytic factor if it is indeed related to the concentration of either arachidonic or linoleic acid in the serum is shown in fig. 58. The triglycerides in the serum are hydrolysed at the muscle surface to their constituent fatty acids and glycerol, some of the fatty acids, arachidonic and linoleic acid in particular would become incorporated into the phospholipid membrane or would enter the cyclooxygenase pathway of prostanoid metabolism thus enhancing muscle catabolism. Phospholipase A_2 activation would result in the release of fatty acids from the phospholipids to be recycled through prostanoid synthesis. The phospholipase A_2 may be stimulated by increased Ca^{2+} levels perhaps through bone dissolution as a consequence of the action of parathyroid hormone or by increased cAMP levels produced as a result of the action of the MAC16 lipolytic factor. Baracos et al, (1986) demonstrated that increased Ca^{2+} levels increased PGE_2 production although this was not equated with increased muscle proteolysis. If the triglycerides are hydrolysed at the muscle surface it is surprising that the fatty acids alone did not elicit an equivalent response - it may be that the cleavage of the fatty acids from the triglyceride is an essential step that is a prerequiste for incorporation into the membrane.

Figure 58.


SKELETAL MUSCLE
TRIGLYCERIDES
ARACHIDONONYL
ARACHIDONONYL
LINOLEONYL
PHOSPHOLIPID INCORPORATION
MEMBRANE BILAYER
PHOSPHOLIPASE A2
ARACHIDONIC ACID, LINOLEIC ACID
CYCLOOXYGENASE PATHWAY
PGE2
AMINO ACID RELEASE

Alternatively the enzyme system may require that the triglyceride presents the fatty acids in a certain array for example with arachidonic acid at position 2. However this is purely speculative.

A recent study Rotman et al (1992) demonstrated that arachidonic acid inhibited protein synthesis in mammalian cells - this led to an experiment on gastrocnemius muscle protein synthesis under the influence of serum from non tumour-bearing and MAC16 tumour-bearing animals with weight loss. Protein synthesis was indeed inhibited by the effect of serum from cachectic animals. Thus the increased levels of arachidonic acid mobilised from adipose tissue during cachexia could contribute to the progression of the disease.

In conclusion the proteolytic factor would appear to be a combination of arachidonic and linoleic acid with its actions largely mediated through the action of the prostanoids - PGE_2 in particular. A number of previous studies suggested that cytokines for example TNFα or IL-1 on their own, or in combination, possibly increased muscle proteolysis through a prostaglandin intermediate (Flores et al, 1989, Clowes et al, 1983). However, the role of cytokines remains controversial and in this case their participation is unlikely as the proteolytic factor is heat stable.

EPA and indomethacin inhibit both the degradative effect of serum from cachectic animals and also inhibit the increase in muscle PGE_2 associated with cachexia. Indomethacin has previously been shown to have an antitumour and anticachectic effect in mice bearing the C57BL/6J tumour (Gelin et al, 1991). EPA and indomethacin both seem worthy of further investigation as anticachectic and antitumour agents.

The decrease in protein synthesis caused by increased arachidonic acid levels is equally important - treatment regimes may be developed that inhibit arachidonic acid levels and thus protein synthesis would be maintained with the possible knock-on effect of the inhibition of protein degradation. For further discussion see appendix.

4.7. Dietary supplementation of the cachectic MAC16 tumour-bearing animal with branched chain amino acids.

4.7.1. Introduction.

The perception of muscle as primarily a locomotive tissue has been substantially modified by a growing knowledge of the role of muscle as a store of fuel to be drawn upon during infection and following trauma. Many clinical conditions result in muscle wasting for example

cardiovascular and lung disease, alcoholism and tuberculosis. The relationship between these diseases and cachexia is discussed in the introduction. The muscle loss is thought to be partially due to a homeostatic imbalance between protein synthesis and degradation - either a depressed synthesis, an increased degradation or a combination of the two (discussed in section 4.0.1.). One possible explanation for this was postulated by Stein (1978) who attributed the abnormal gluconeogenesis seen in cancer patients to the tumour's greater avidity for certain essential amino acids - this left the host with the problem of disposing of the remainder of the amino acids. Removal of one amino acid would lead to a depression of host protein synthesis, since normal protein synthesis requires a full complement of amino acids. In a study of tumour-bearing rats (Walker carcinoma 256) Krause et al (1979) found decreased plasma levels of serine, glycine, aspartate and hydroxyproline only in tumour-bearing animals. Similarly Levin et al (1983) found that there were significantly lower arterial levels of 12 out of 19 amino acids in the cachectic cancer patient when compared with tuberculosis patients with comparable weight loss. In the MAC16 tumour-bearing animal the plasma level of all amino acids decreased with increasing tumour size except for taurine. The maximum decrease of 54% was observed for valine and isoleucine. Most of the other amino acids were present in tumour-bearing mice at only 60% of the level

found in non tumour-bearing animals (Beck and Tisdale, 1989). These are presumerably reflections of enhanced tumour sequestration of certain amino acids.

The branched chain amino acids leucine, isoleucine and valine are essential amino acids for the human and must be supplied from the diet since we have no metabolic capacity to synthesise them. In cachexia there is an increased concentration of alanine in the blood which is dependent upon the oxidation of branched chain amino acids in muscle (Williams and Matthaei, 1981). Alanine is the main amino acid substrate for hepatic gluconeogenesis - the rate being positively related to the concentration of alanine in the blood. The alanine cycle also allows the metabolism of amino acids by peripheral tissues without increasing blood ammonia and provides substrate to glucose dependent tissues during periods of limited supply. In muscles, stimulated oxidation of branched chain amino acids complements the release of alanine, the carbon skeleton of the branched chain amino acids serve as an energy substrate for muscle cells - and the amino group for the transamination of pyruvate to alanine (Buse et al, 1972). The uncontrolled branched chain amino acid oxidation that may lead to muscle loss in cancer cachexia is proposed by Williams and Matthaei (1981) to be partially due to tumour induced retrodifferentiating changes in the liver - resulting in massive decreases in the activity of alanine

aminotransferase. This is the only enzyme capable of deaminating alanine such that its carbon skeleton (pyruvate) is unavailable for gluconeogenesis. In addition an insufficient concentration of ketone bodies in the blood, hypoinsulinaemia and tissue insulin resistance (Kisner et al, 1978) may subvert the ability of the host to regulate branched chain amino acid oxidation. Thus the branched chain amino acids are thought to play a unique physiological role as a substrate and also as a regulator of carbohydrate and protein metabolism. In post operative patients the infusion of leucine, isoleucine and valine were found to be as effective or more effective than a more balanced amino acid solution in preventing muscle catabolism (Freund et al, 1979). Schaur et al (1980) demonstrated that the continuous administration of physiological doses of the branched chain amino acids to Yoshida sarcoma bearing rats caused a significant 32% and 33% increase in survival time and decrease in tumour growth respectively - however the shift of nitrogen balance to negative values during the cachectic stage was delayed but not prevented. In 1989 Hunter et al showed that cachectic patients with intra-abdominal adenocarcinoma treated with branched chain amino acids as the protein component of parenteral nutrition had a reduction in protein oxidation and an increase in protein synthesis and fractional albumin synthetic rate.

In vitro branched chain amino acids have a stimulatory effect on muscle protein synthesis (Buse and Reid, 1975, Fulks et al, 1975) although this effect was not observed in vivo. Protein degradation in incubated or perfused skeletal muscle was also inhibited. The anabolic effect of the plasma amino acids could be accounted for by the presence of branched chain amino acids alone and leucine in particular. These findings are of particular interest because they are unique to muscle - in liver and fibroblasts branched chain amino acids have no such effect. Furthermore these effects can be demonstrated with leucine at concentrations ranging between 0.1 and 0.5mM which is the range over which blood concentrations of leucine change in the fed and fasted states. Leucine seems to serve as an alternative to using glucose or glycogen as a source of acetyl CoA. Thus leucine seems to be a physiological regulator of protein balance in muscle acting similarly to insulin (Goldberg and Tischler, 1981).

The inhibitory effect of leucine on protein breakdown can be induced with α-ketoisocaproate (ketoleucine) and blocked with inhibitors of leucine transaminase (Tischler et al, 1982). Protein synthesis is not affected by these treatments but is dependent on the concentration of free leucine (Goldberg and Tischler, 1981). Leucine would appear to have a special role in maintaining homeostasis during starvation and its removal by a tumour may give rise

to some of the symptoms of cachexia. The aim of this section is to identify the effect the branched chain amino acids leucine, isoleucine and valine have on cachexia associated with the MAC16 murine adenocarcinoma.

4.7.2. Results.

The treated and non-treated controls and MAC16 animals were given the branched chain amino acids leucine 23.24mM, isoleucine 14.53mM, and valine 22.76mM in their drinking water. Treatment of the MAC16 animals was initiated 14 days after implantation when cachexia became apparent (mice had lost 6-8% body weight). Branched chain amino acid treatment did not significantly reduce the weight loss seen in animals bearing the MAC16 tumour (Fig. 59B). The weight loss in the non-treated and treated MAC16 groups was not accompanied by a decrease in food and water intake when compared to the non-treated and treated control groups (Fig. 60A, 60B, 61A, 61B). The experiment was terminated after 6 days because some animals were reaching the limits of the terms agreed by the coordinating committee on cancer research of the United Kingdom for the welfare of animals with neoplasms. The branched chain amino acid treatment appeared to marginally stimulate tumour growth although this was not significant (Fig.62A). Lactate, pyruvate, glucose, and albumin levels were not significantly altered by branched chain amino acid treatment (Fig.62B). The

Figure 59A.
The effect of branched chain amino acid treatment on non tumour-bearing (NTB) mouse weight.

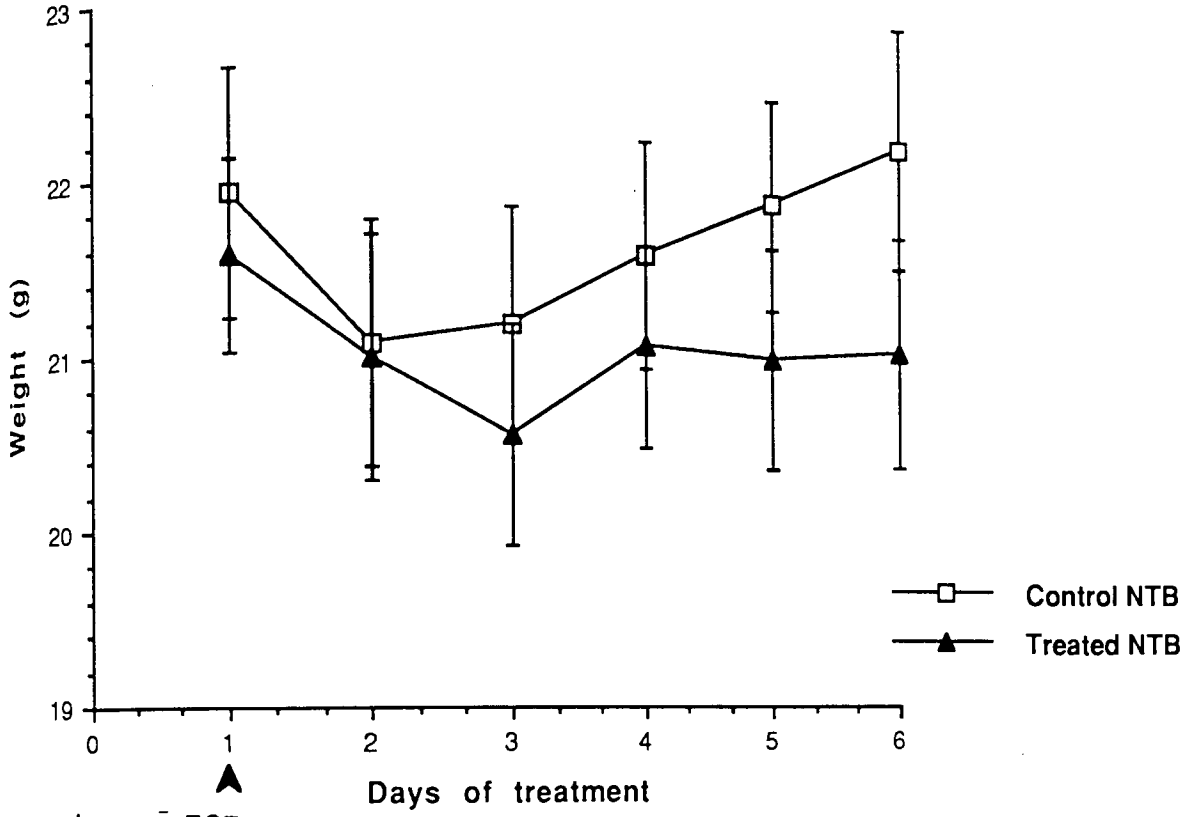
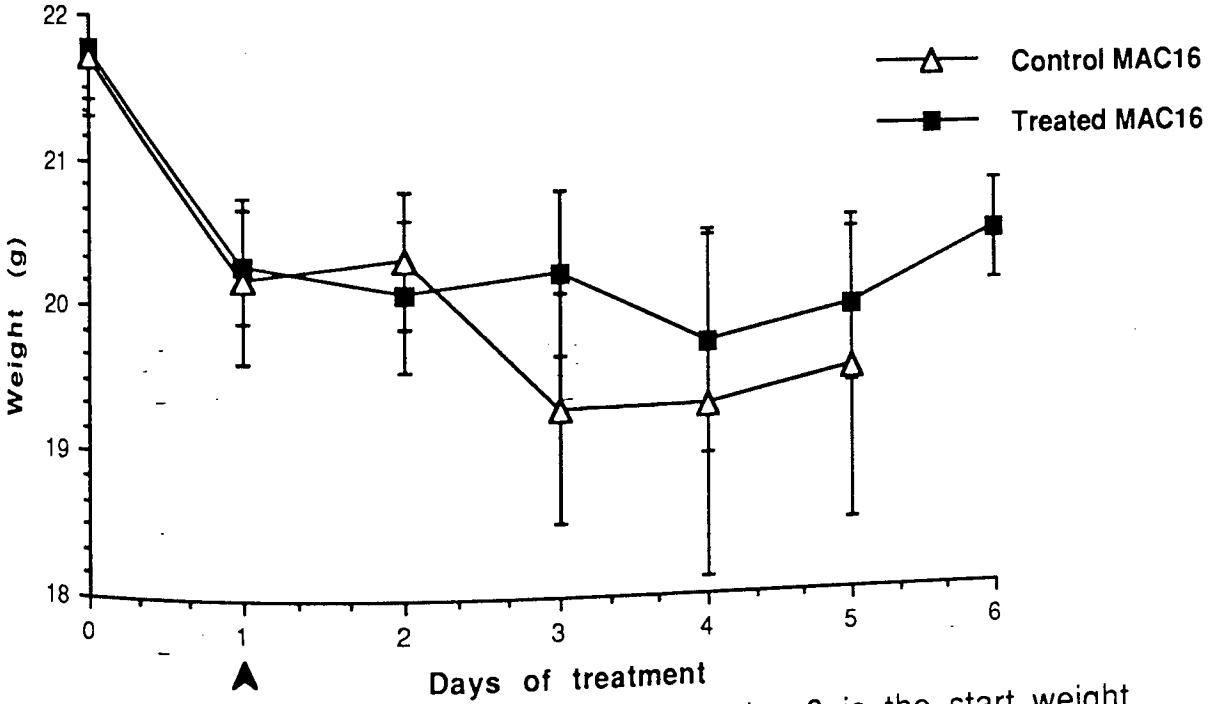


Figure 59B.
The effect of branched chain amino acid treatment on the weight loss produced by the MAC16 adenocarcinoma.



Day 1 is the start of the treatment, day 0 is the start weight prior to tumour implantation. Results are expressed as the mean ±SEM for 6 animals per group. There were no significant differences between the treated and control groups.

Figure 60A.
The food and water intake of NTB mice.

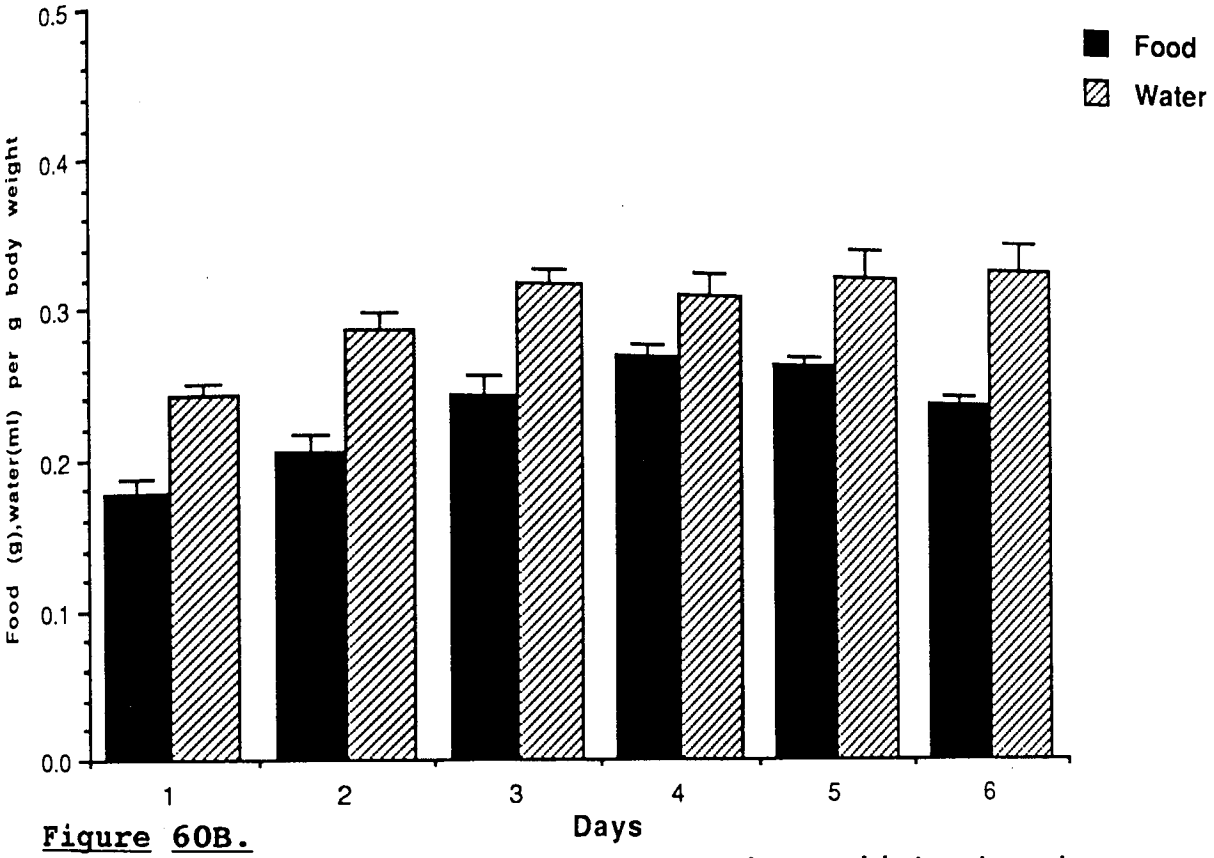
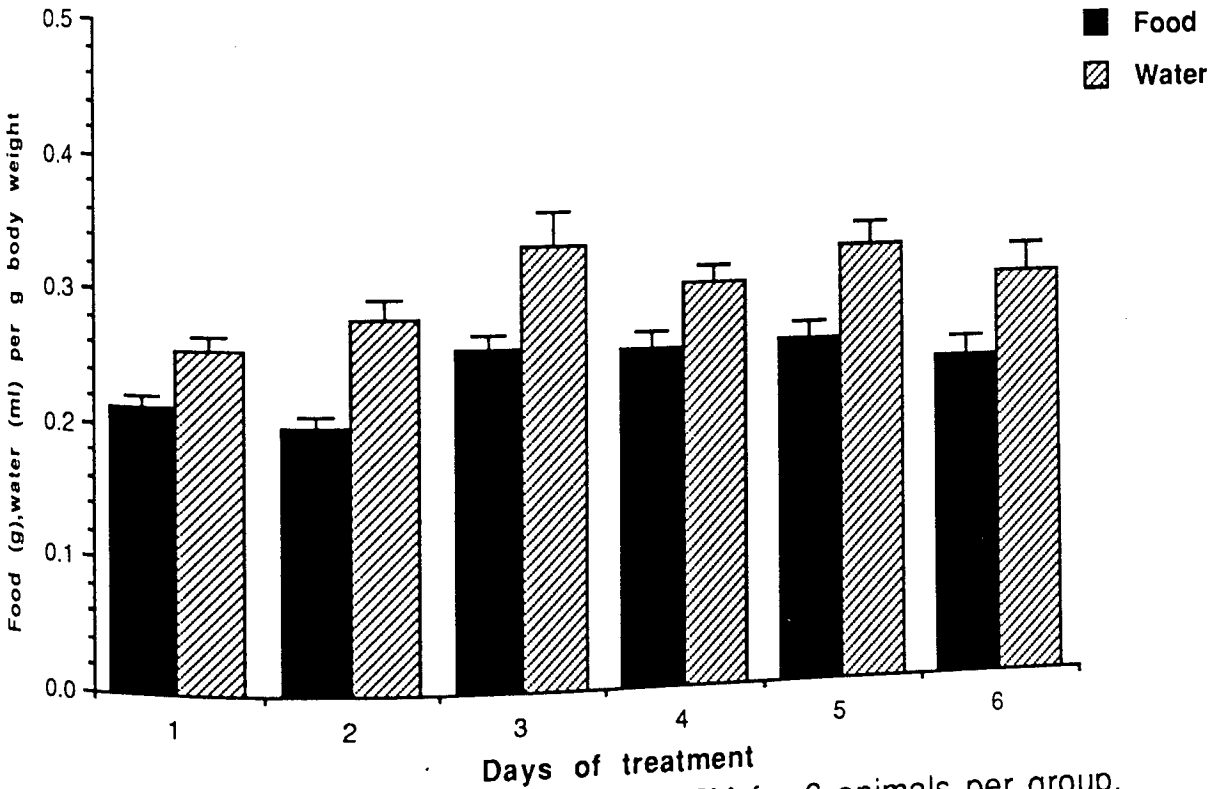


Figure 60B.
The effect of branched chain amino acid treatment on the food and water intake of NTB mice.



Each bar represents the mean ± SEM for 6 animals per group.
There were no significant differences between groups.

Figure 61A.

The food and water intake of cachectic MAC16 tumour-bearing mice.

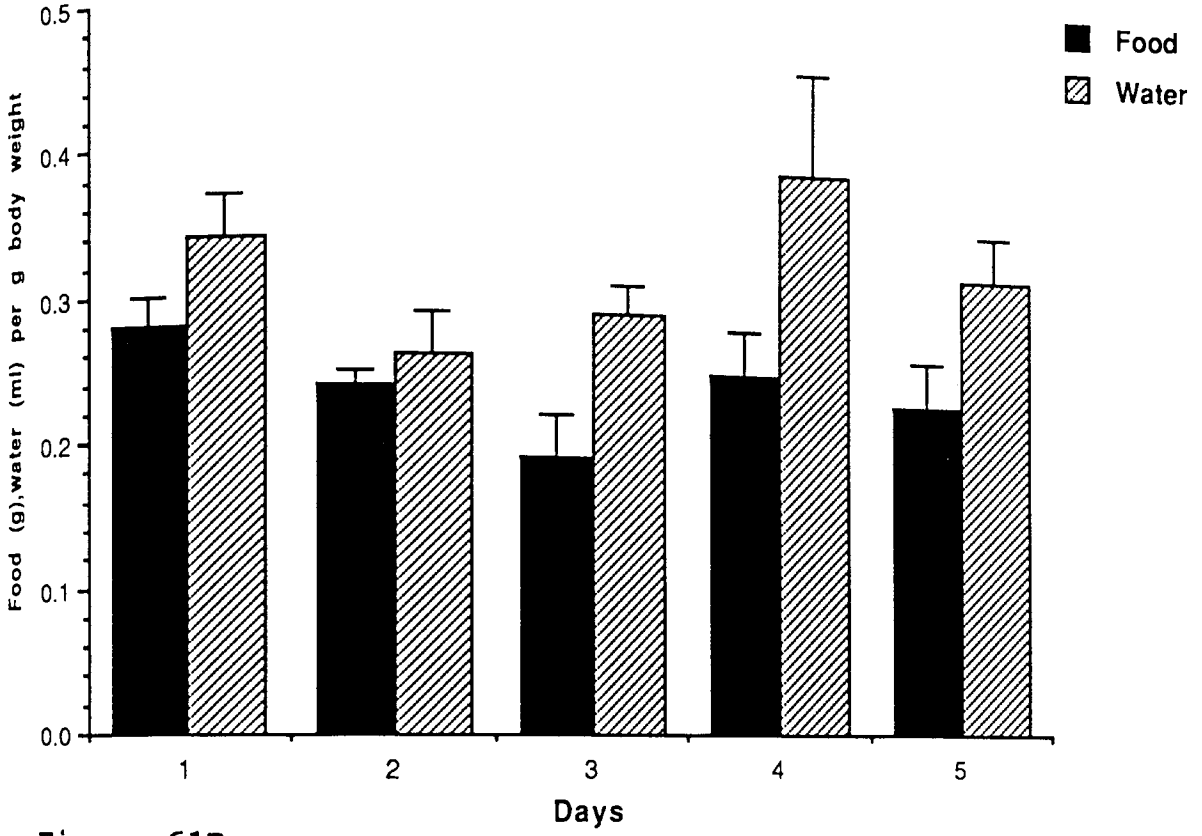
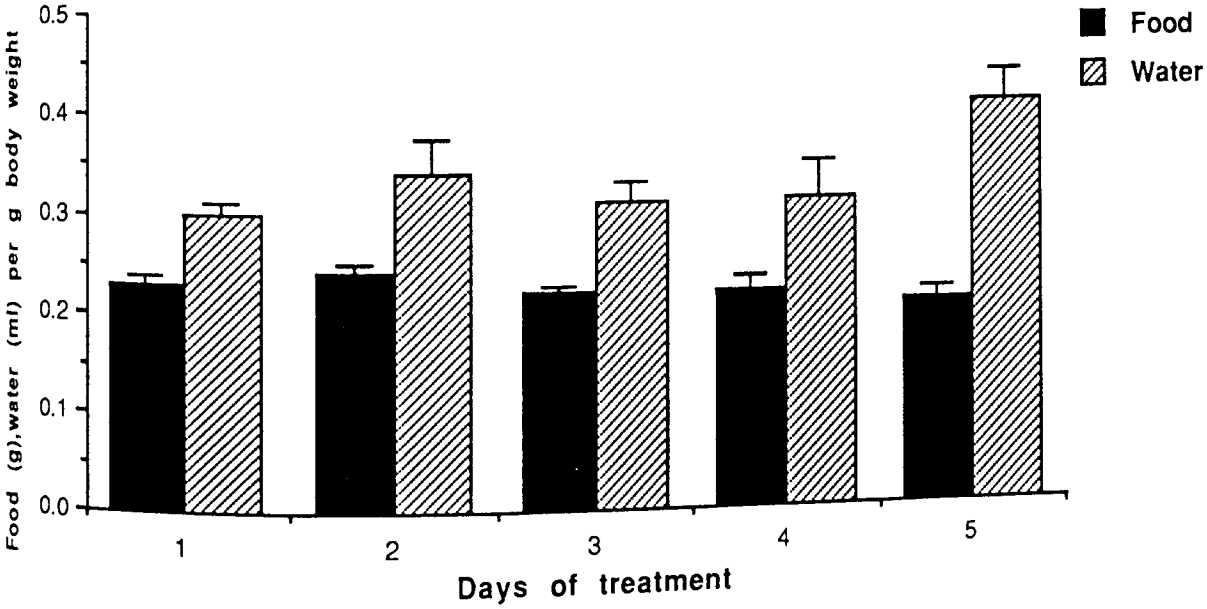


Figure 61B.

The effect of branched chain amino acid treatment on the food and water intake of cachectic MAC16 tumour-bearing mice.



Each bar represents the mean ± SEM for 6 animals per group. There were no significant differences between the control and treated MAC16 tumour-bearing groups or between the MAC16 tumour-bearing and NTB control and treated groups.

Figure 62A.

The effect of branched chain amino acid treatment on the growth of the MAC16 adenocarcinoma.

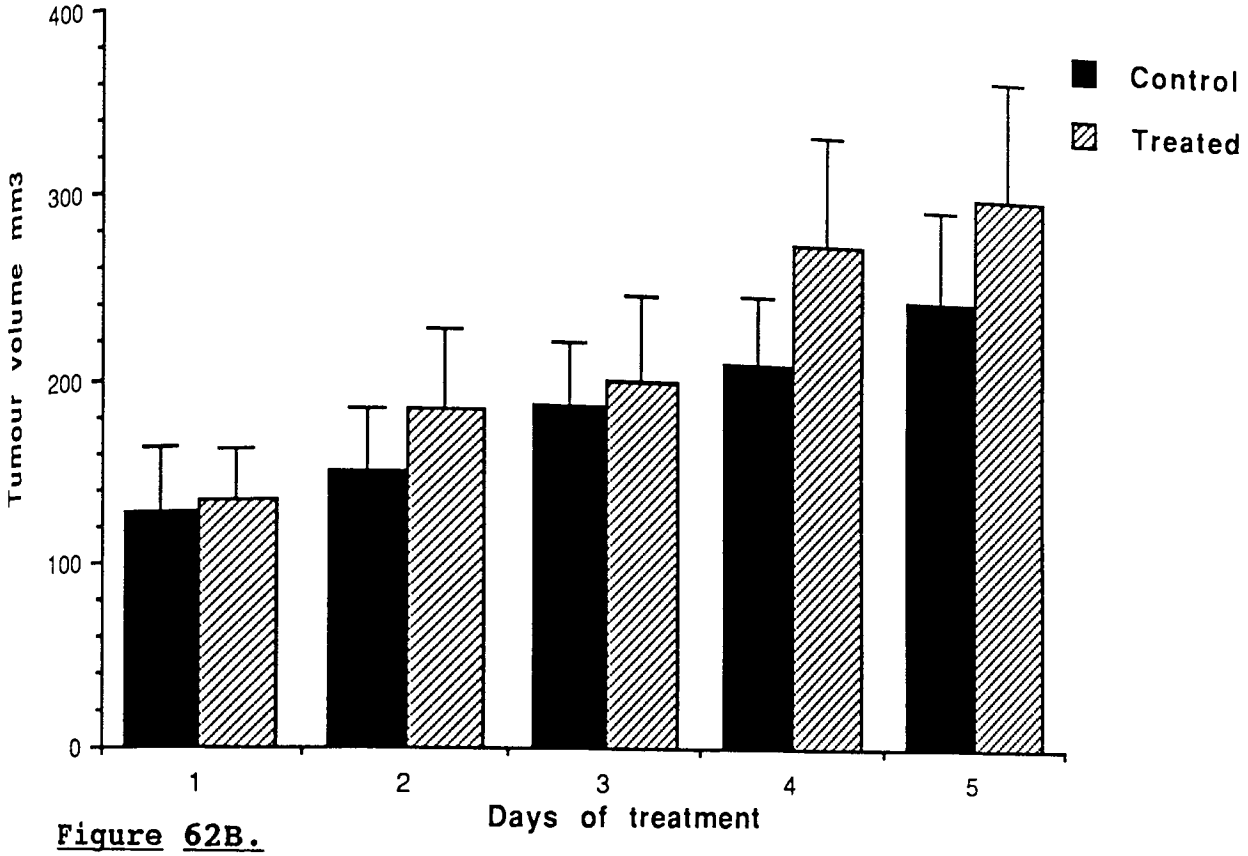
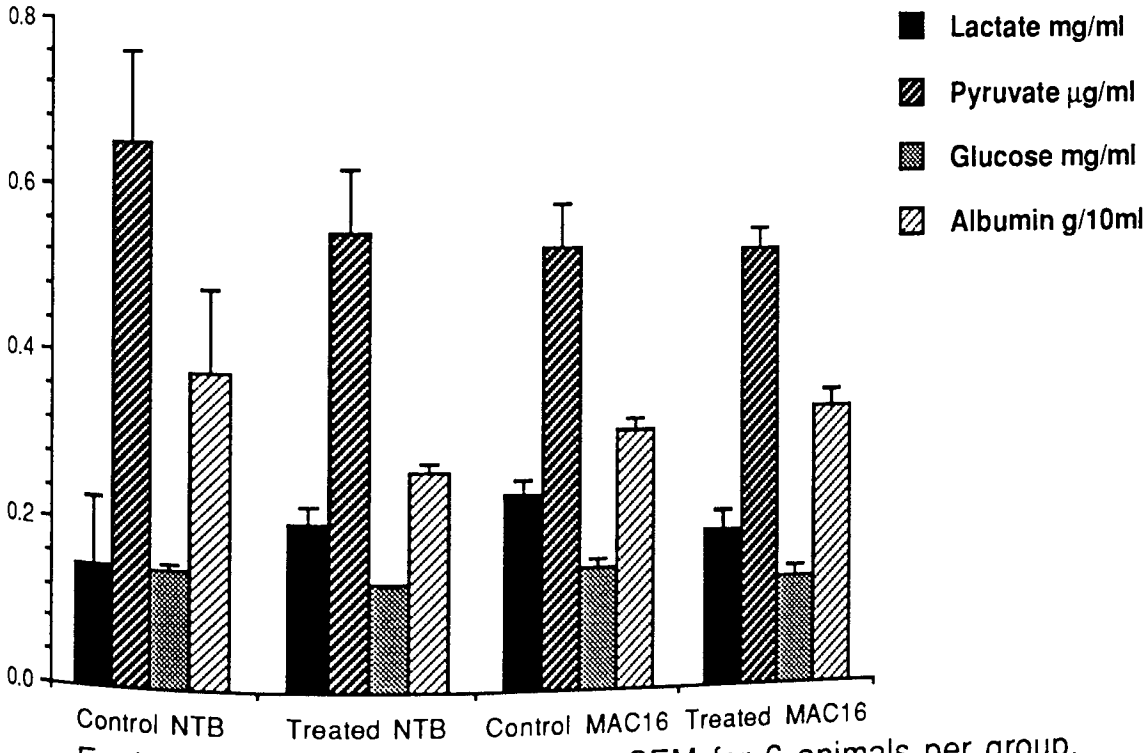


Figure 62B.

The effect of branched chain amino acid treatment on blood lactate, pyruvate, glucose and albumin levels.



Each bar represents the mean ± SEM for 6 animals per group. There were no significant differences between groups.

Figure 63A.
The body composition of NTB mice.

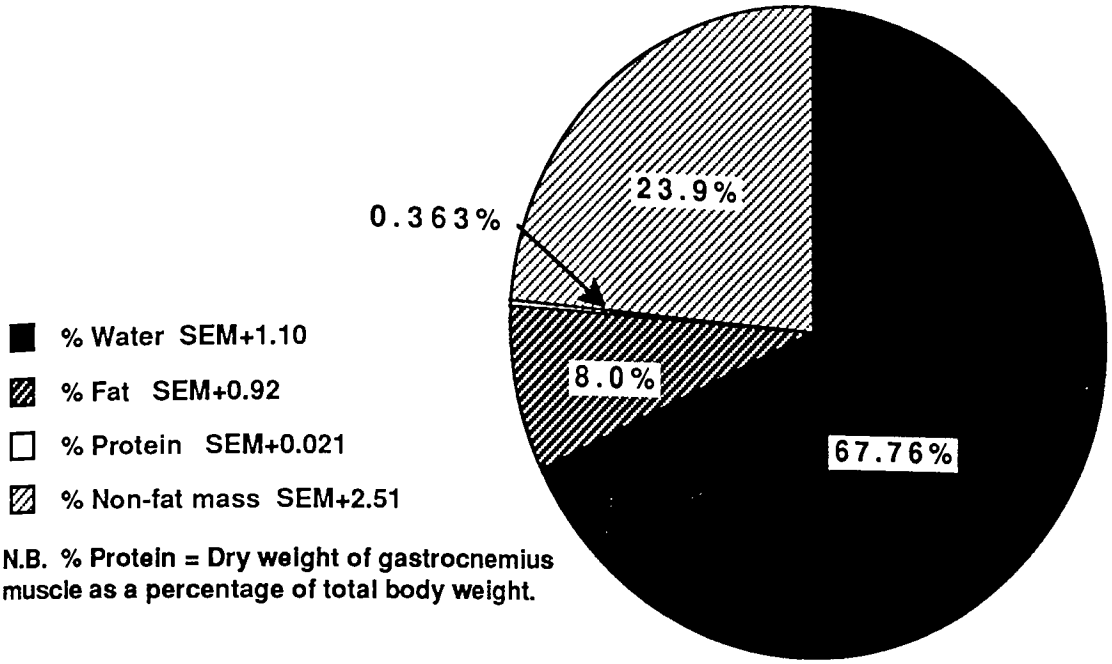
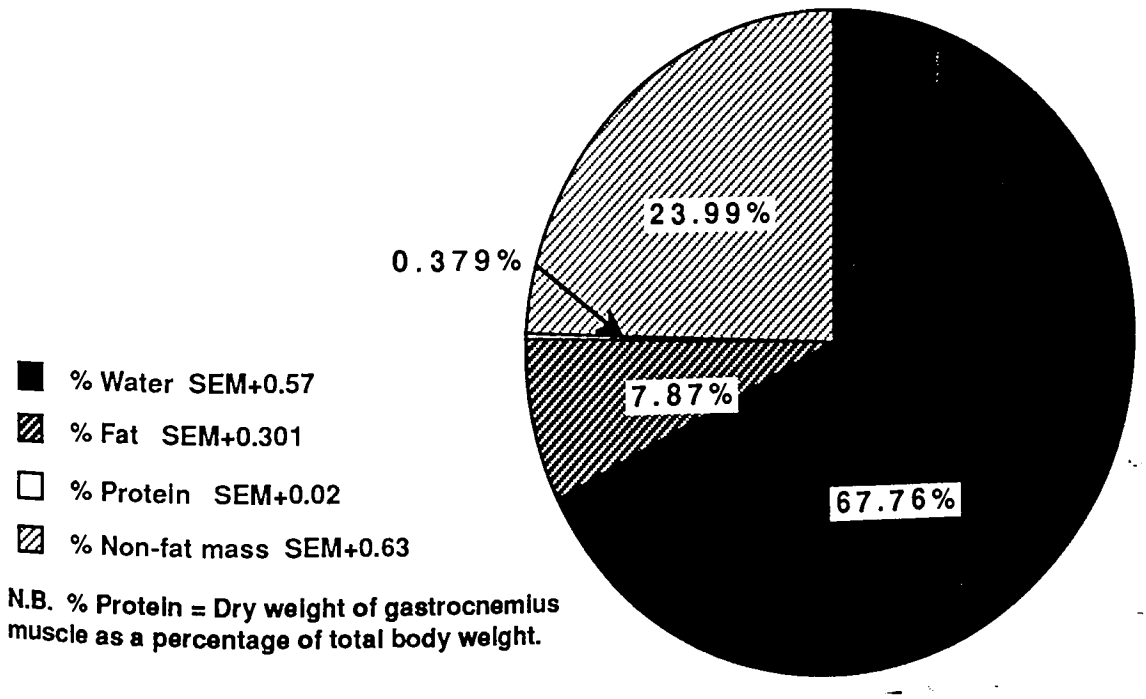


Figure 63B.
The effect of branched chain amino acid treatment on the body compostion of NTB mice.



Results are expressed as the mean ± SEM for 6 animals.

Figure 64A.

The effect of branched chain amino acid treatment on the body composition of cachectic MAC16 tumour-bearing mice.

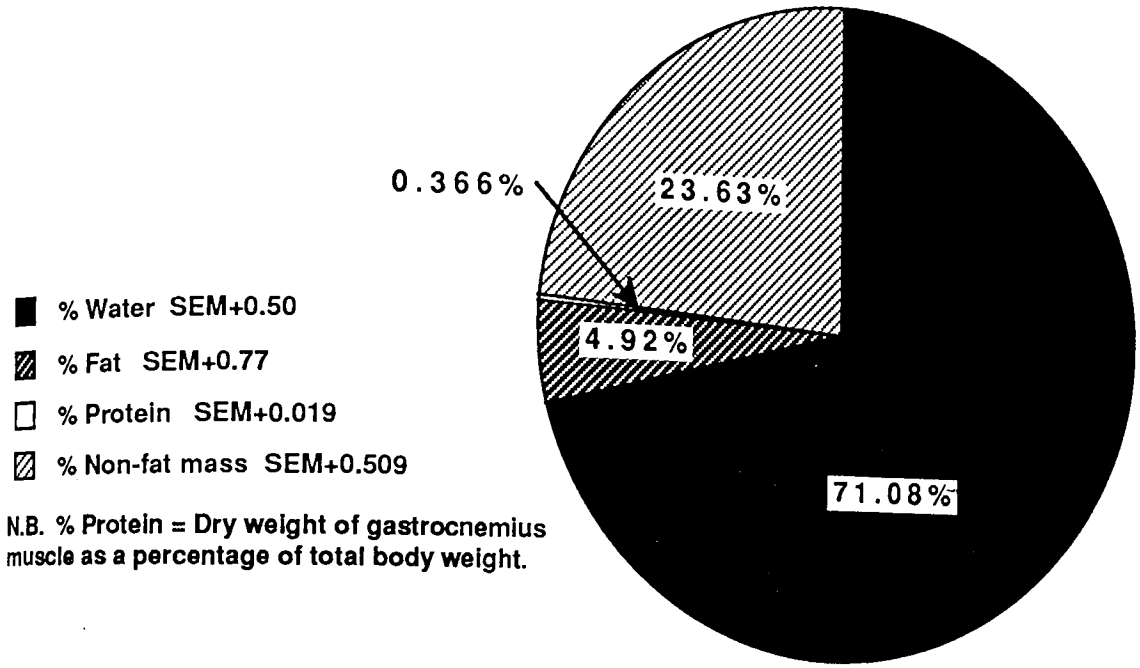
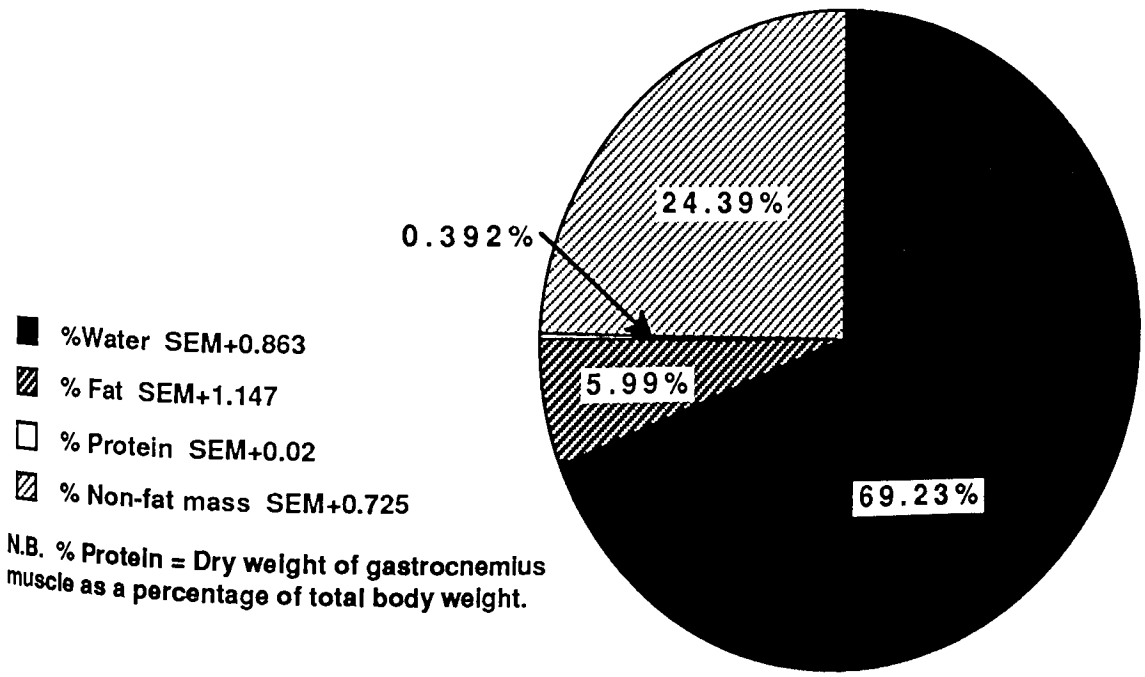


Figure 64B.

The body composition of cachectic MAC16 tumour-bearing mice.



Results are expressed as the mean ± SEM for 6 animals

analysis of the body compartments of the four groups is shown in figs. 63A, 63B, 64A, 64B. There are no significant differences between non-treated and treated control groups. There is a significant decrease in the fat content of the non-treated and treated MAC16 groups when compared to the control group and this is accompanied by an increase in body water content that is not significant. The protein compartment as calculated as percentage dry weight of gastrocnemius and thigh of total body weight remained unchanged in all groups.

4.7.3. Discussion.

The administration of branched chain amino acids to cachectic MAC16 tumour bearing animals did not in this case increase survival time or decrease tumour growth as had been previously shown. However, there is a slight trend towards an increase in mouse body weight in the treated MAC16 group. The increase in weight appears to be largely from water retention as seen in the body composition analysis. It has been shown that it is extremely difficult to replenish lean body tissue stores and any weight gained is mainly due to an accumulation of large quantities of intracellular fluid and fat (Cohn et al, 1982, Shamberger, 1984). Indeed Nixon et al (1981) suggest that the widespread metabolic aberations in the cachectic host decrease and even block the ability of the organism to

utilise nutrients for the synthesis of normal lean body tissue. Protein depletion is not a prominent feature of the MAC16 cachectic animal in this experiment - this is possibly due to the fact that the thigh and gastrocnemius muscles only represent approximately 0.4% of the total body weight - consequently changes are difficult to detect. Also since muscle loss usually follows fat breakdown it was not practical to achieve this boundary due to Home Office regulations. In this experiment large tumour size and necrosis pre-empted the termination of the experiment rather than excessive weight loss. There is also evidence that there appears to be a protein conservation mechanism in operation when MAC16 tumour-bearing animals lose 3-4 g in body weight (see section 4.0.3.). The slight increase in tumour size is not uncommon - Balducci and Hardy (1985) suggest that with the metabolic derangements associated with cachexia hyperalimentation is more likely to feed the tumour than the patient. This view is supported by DeWys (1985) who warns against the use of excessively aggressive hyperalimentation in case this stimulates tumour growth. Indeed the tumour has the ability to preferentially sequester amino acids from the plasma amino acid pool more efficiently than do normal cells. Wiseman and Ghadially (1955) demonstrated that sarcoma cells could concentrate mono-amino and di-amino carboxylic acids. In addition if the MAC16 murine adenocarcinoma is analogous to an ascites carcinoma cell line (described by Lazo in

1981)in its leucine dependency there will be a drain on host leucine sources proportional to the size of the tumour. This creates a concentration gradient between the muscle, the free amino acid pool and the tumour thus causing a net loss of leucine. The consequent deprivation of the host of essential amino acids leads to the waste of other amino acids and to the net loss of less vital proteins in the organism either by stopping their synthesis or by degradation as a compensatory effect. Thus an additional exogenous supply of leucine could theoretically promote tumour growth. The administration of branched chain amino acids did not affect food and water intake in the treated groups. It is interesting to note that the amount of protein an animal will voluntarily consume depends on its ability to metabolise amino acids within a short time period. Anderson et al (1968) demonstrated that the food intake of rats fed a high protein diet was initially depressed when enzyme activity was low and plasma amino acid concentration was high. Food intake rose subsequently as enzyme activity increased and plasma amino acids decreased. However, in the same animals the plasma concentration of branched chain amino acids continued to increase suggesting that the degradative enzymes in this case respond more slowly, or to a lesser extent, or that the branched chain amino acids are transported to the tissues more slowly.

The consistancy of the lactate, pyruvate, glucose and albumin levels suggest that the MAC16 tumour-bearing animals are maintaining metabolic homeostasis. The slight but insignificant rise in the MAC16 lactate level is probably due to an increased Cori cycle activity with concomitant increase in lactate production. Holroyde et al (1975) demonstrated that patients with progressive weight loss had an increased Cori cycle activity. Hypoalbuminaemia is not a prominent feature and this again suggests that the MAC16 animals are not in a protein depletive state. Costa (1963) correlated hypoalbuminaemia to the extent of the cancer and he attributed this to a decrease in protein synthesis.

From the results it can be concluded that the dietary supplementation of the cachectic MAC16 tumour-bearing mouse with branched chain amino acids is ineffective at reversing cachexia. The major limiting factor in the MAC16 model of cachexia in this case, is the fact that animals appear to lose protein fairly late on in the progression of cachexia and a substantial negative nitrogen balance is not achieved.

CHAPTER 5: CONCLUSION.

In the MAC16 tumour-bearing animal there was a significant reduction in carcass nitrogen between 16-30% weight loss and this was associated with a reciprocal progressive increase in tumour nitrogen content. The loss of carcass nitrogen was accompanied by a concomitant decrease in gastrocnemius muscle weight and nitrogen content and also by a decrease in liver nitrogen content. The loss of gastrocnemius muscle throughout the progression of cachexia was associated with a 60% decrease in rate of protein synthesis and a 240% increase in the rate of protein degradation. Thus if this effect was translated to the whole body a decreased rate of protein synthesis and an increased rate of protein degradation would exacerbate the cachectic state.

The MAC16 adenocarcinoma has been shown to produce a circulatory catabolic factor that has a proteolytic activity in the host (Beck et al, 1990). A serum factor was found in cachectic MAC16 tumour-bearing animals that stimulated protein degradation in isolated gastrocnemius muscle. This proteolytic factor was not found in serum from non tumour-bearing animals or in serum from animals bearing the related MAC13 tumour that does not produce cachexia. The level of the proteolytic factor in serum can be shown to increase with increasing weight loss up to 20% weight loss and thereafter decreases. It was proposed that the factor may be responsible for the initiation of the

increased rate of protein degradation, but may not contribute to its maintenance at high weight loss. It was interesting to note that there was a linear relationship observed between both the serum and urinary lipolytic activity and weight loss in the cancer patient when weight loss did not exceed 20%. There was a similar relationship demonstrated in the MAC16 tumour-bearing animal where weight loss was paralleled by a corresponding increase in serum lipolytic activity which peaked at 16% weight loss (Groundwater et al, 1990).

The enzyme guanidinobenzoatase is found in mouse serum and cleaves the substrate 4-methylumbelliferyl-p-guanidinobenzoate to yield the fluorescent product methylumbelliferone. As the enzyme was associated with the invading edges of malignant tumours it was proposed that it may be systemically released and thus mediate protein breakdown in the cachectic MAC16 tumour-bearing animal. However, the enzyme was also found in the serum of non tumour-bearing animals and humans as well as in the tumour-bearing state. The ubiquitous nature of guanidinobenzoatase thus precluded it from being associated with the development of cachexia. The identity of the serum enzyme was sought and was found to be closely related to, but distinct from, the tumour cell-bound guanidinobenzoatase. EPA and BZAR were found to inhibit tumour cell-bound guanidinobenzoatase and thus may be

important in preventing metastasis.

The nature of the proteolytic factor was further investigated and was found to be heat stable and not a serine protease - thus it was distinct from the factor described by Beck et al (1990). The proteolytic factor was inhibited by indomethacin and EPA - suggesting the involvement of the prostanoids. This was further substantiated by the fact that the serum factor caused a significant increase (50%) in the PGE_2 content of isolated gastrocnemius muscle. The protein degradative effect of serum from cachectic MAC16 tumour-bearing animals was largely mimicked by the addition of the triglycerides, triarachidonin and trilinolein, to serum from non tumour-bearing animals. Thus it was proposed that arachidonic and linoleic acid are released from the triglycerides at the muscle level where they are incorporated into membrane phospholipids or channelled through the cylclooxygenase pathway to form PGE_2. The activity of phospholipase A_2 governs the release of the fatty acids from the phospholipids fuelling PGE_2 synthesis and protein degradation. Arachidonic acid levels have been shown to be increased (Hudson, 1992, unpublished results) in cachectic MAC16 tumour-bearing animals as a result of mobilisation of lipid stores. Protein synthesis in mammalian cells has been shown to be inhibited by arachidonic acid - similarly protein synthesis in the

isolated gastrocnemius muscle was inhibited under the influence of serum from cachectic MAC16 tumour-bearing animals - again emphasizing the importance of serum fatty acid/triglyceride levels in the regulation of protein turnover. The "proteolytic" factor requires further investigation - indeed the factor could be a metabolic consequence of the action of the lipolytic factor that is currently being isolated and sequenced.

Branched chain amino acid treatment has been shown to minimise protein catabolism in the post-operative traumatised patient (Freund et al, 1979). In the cachectic MAC16 tumour-bearing animal branched chain amino acid treatment was ineffective in reversing the cachectic state. This was largely due to the late onset of a negative nitrogen balance.

The polyunsaturated fatty acid EPA however, has been shown to have a pronounced anticachectic/antitumour effect in animals bearing the MAC16 tumour (Tisdale and Beck, 1991). The maintenance of host body weight in animals treated with EPA was associated with a 60% decrease in the rate of skeletal muscle protein degradation and a 16% increase in protein synthesis which was not significant. EPA inhibited the proteolytic factor, inhibited the prostaglandin content of muscle challenged with serum from cachectic MAC16 tumour-bearing animals and muscle from EPA treated animals

was particularly resistant to the degradative effect of the proteolytic factor.

EPA is to currently undergo a Cancer Research Campaign phase one clinical trial as an anticachectic and antitumour agent.

REFERENCES.

AISENBERG, A.C., and MORNS, H.P. (1961) Energy pathways of hepatoma no. 5123. Nature, 191, 1314-1315.

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. and WATSON, J.D. (1983) The molecular biology of the cell. Garland publish. Inc. London, 356-359.

ANDERSON, H.L., BENEVENGA, N.J. and HARPER, A.E. (1968) Associations among food and water intake, serine dehydratase and plasma amino acids. Am.J.Phys., 214(5), 1008-1013.

ARGILES, J.M. and AZCON-BIETO, J. (1988) The metabolic environment of cancer. Mol.Cell.Biochem., 81, 3-17.

ASHFORD, A.J. and PAIN, V.M. (1986) Effect of diabetes on the rates of synthesis and degradation of ribosomes in rat muscle and liver in vivo. J.Biol.Chem., 261(9), 4059-4065.

BAILLE, C.A., ZINN, W.M. and MAYER, J. (1970) Effects of lactate and other metabolites on food intake of monkeys. Am.J.Phys., 219, 1606-1613.

BAKER, N., SANDBORG, C., MORNS, D. and OOKHTENS, M. (1977) Competition for host essential and non-essential fatty acids in Ehrlich ascites carcinoma in mice. Cancer Res., 37, 2218-2225.

BALDUCCI, L. and HARDY, C. (1985) Cancer and malnutrition - a critical interaction: A review. Am.J.Haematology, 18, 91-103.

BARACOS, V.E. and GOLBERG, A.L. (1986) Maintenance of normal length improves protein balance and energy status in isolated rat skeletal muscles. Am.J.Physiol., 251, C588-C595.

BARACOS, V.E., GREENBERG, R.E. and GOLDBERG, A.L. (1986) Influence of calcium and other divalent cations on protein turnover in rat skeletal muscle. Am.J.Physiol., 250, E702-E709.

BARACOS, V.E., RODEMANN, H.P., DINARELLO, C.A. and GOLDBERG, A.L. (1983) Stimulation of muscle protein degradation and prostaglandin E_2 release by leukocytic pyrogen (Interleukin-1). New Eng.J.Med., 308, 353-358.

BARNETT, J.G. and ELLIS, S. (1987) Prostaglandin E_2 and the regulation of protein degradation in skeletal muscle. Muscle Nerve, 10, 556-560.

BECK, S.A., MULLIGAN, H.D. and TISDALE, M.J. (1990) Lipolytic factors associated with murine and human cancer cachexia. J.Natl.Cancer Inst., 82, 1922-1926.

BECK, S.A. and TISDALE, M.J. (1987) Production of lipolytic and proteolytic factors by a murine tumour - producing cachexia in the host. Cancer Res., 47, 5919-5923.

BECK, S.A. and TISDALE, M.J. (1989) Effect of insulin on weight loss and tumour growth in a cachexia model. Br.J.Cancer. 59, 677-681.

BECK, S.A. and TISDALE, M.J. (1989) Nitrogen excretion in cancer cachexia and its modification by a high fat diet in mice. Cancer Res., 49, 3800-3804.

BELIZARIO, J.E., KATZ, M., and RAW, I. (1991) Bioactivity of skeletal muscle proteolysis-inducing factors in the plasma proteins from cancer patients with weight loss. Br.J.Cancer. 63, 705-710.

BEYNON, R.J. and KAY, J. (1978) The inactivation of native enzymes by a neutral proteinase from rat intestinal muscle. Biochem.J., 173, 291-298.

BIBBY, M.C., Double, J.A., ALI, S.A., FEARON, K.C.H., BRENNAN, R.A. and TISDALE, M.J. (1987) Characterisation of a transplantable adenocarcinoma of the mouse colon producing cachexia in recipient animals. J.Natl.Cancer Inst., 78, 539-546.

BIRD, J.W., CARTER, J.H., TRIEMER, R.E., BROOKS, R.M. and SPANIER, A.M. (1980) Proteinases in cardiac and skeletal muscle. Fed.Proc.Fed.Am.Soc.Exp.Biol., 39, 20-25.

BJORNTORP, F. (1966) Effect of ketone bodies on lipolysis in adipose tissue in vitro. J.lipid Res., 7, 621-626.

BREM, H., LANGER, R., FALTERMAN, K., KLEIN, M. and FOLKMAN, J.(1976) Inhibition of a cartilage factor that inhibits tumour neovascularisation. Science, 193, 70-71.

BRENNAN, M.F. (1977) Uncomplicated starvation versus cancer cachexia. Cancer Res., 37, 2359-2364.

BUSE, M.G., BIGGERS, J.F., FRIDERICI, K.H. and BUSE, J.F. (1972) Oxidation of branched chain amino acids by isolated hearts and diaphragms of the rat. J.Biol.Chem., 247(24), 8085-8096.

BUSE, M.G. and REID, S.S. (1975) Leucine - a possible regulator of protein turnover in muscle. J.Clin.Invest., 56, 1250-1261.

BUZBY, G.P., MULLEN, J.L., STEIN, T.P., MILLER, E.E., HOBBS, C.L. and ROSATO, E.F. (1980) Host-tumour interaction and nutrient supply. Cancer, 45, 2940-2948.

CAMPBELL, N.R., READE, P.C.and RADDEN, B.G. (1974) Effect of cysteine on the survival of mice transplanted with malignant thymoma. Nature, 251, 158-159.

CERAMI, A., IKEDA, Y., LETRANG, N., HOTEZ, P.J. and BEUTLER, B. (1985) Weight loss associated with an endotoxin-induced mediator from peritoneal macrophages : the role of cachectin / tumour necrosis factor. Immunology lett., 11, 173-177.

CHUTE, C. and GREENBERG, R. (1985) Presenting conditions of 1539 population based lung cancer patients by cell type and stage in New Hampshire and Vermont. Cancer, 56, 2107-2111.

CLARK, C.M. and GOODLAD, G.A.J. (1971) Depletion of proteins of phasic and tonic muscles in tumour-bearing rats. Europ.J.Cancer, 7, 3-9.

CLOWES, G.H.A., GEORGE, B.C., VILLEE, C.A. and SARAVIS, C.A. (1983) Muscle proteolysis induced by a circulating peptide in patients with sepsis or trauma. New Eng.J.Med., 308, 10, 545-551.

COHN, S.H., VARTSKY, D., VASWANI, A.N., SAWITSKY, A., RAI, K., GARTENHAUS, W., YASUMURA, S. and ELLIS, E.J. (1982)

Changes in body composition of cancer patients following combined nutritional support. Nutrition and cancer, 4(2), 107-119.

COLEMAN, P.L. and GREEN, D.J. (1981) A coupled photometric assay for plasminogen activator. Methods in enzymology, 80, 409-415.

COLEMAN, P.L., LATHAM, H.G.T., SHAW, E.N. (1986) A sensitive assay for guanidinobenzoatase. Methods in enzymology. 45, 12-26.

CONYERS, R.A.J., NEED, A.G., DURBRIDGE, T. (1979) Cancer, ketosis and parenteral nutrition. Med.J.Australia, 1, 398-399.

COSTA, G. (1963) Cachexia, the metabolic component of neoplastic disease. Progr.Exp.Tumour Res., 3, 321-369.

CURRERI, P.W. and LUTERMAN, A. (1978) Nutritional support of the burned patient. Surgical Clinics North Am., 58(6), 1151-1156.

DABBOUS, M.K., ROBERTS, A.N. and BRINKLEY, B. (1977) Collagenase and neutral protease activities in cultures of rabbit VX-2 carcinoma. Cancer Res., 37, 3537-3544.

DAHLMANN, B., BLOCK, I., KUEHN, L., RUTSCHMANN, M. and REINAUER, H. (1982) Febs.Letters, 138, 88-90.

DAHLMANN, B. and REINAUER, H. (1981) In : Streptozotocin - fundamentals and therapy. Elsevier, North Holland. pp. 195-205.

DANERYD, P.L., HAFSTROM, L.R., KARLBERG, I.H. (1990) Effects of spontaneous exercise on experimental cancer anorexia and cachexia. Europ.J.Cancer, 26(10), 1083-1088.

DE WYS, W. (1978) Changes in taste sensation and feeding behaviour in cancer patients: a review. J.Human Nutr., 32, 447-453.

DE WYS, W. (1985) Management of cancer cachexia. Seminars in Oncology, 12(4), 452-460.

DE WYS, W. (1986) Weight loss and nutritional abnormalities in cancer patients: Incidence, severity and significance. In: Clinics in oncology, Vol.5, (2). Nutritional support for the cancer patient. (Eds. Calman, K.C. and Fearon, K.C.) W.B. Saunders Co., London. pp. 251-263.

DONALDSON, S.S. and LENON, R.A. (1979) Alterations of nutritional status - impact of chemotherapy and radiation

therapy. Cancer, 43, 2036-2052.

DRESDEN, M.H., HEILMAN, S.A. and SCHMIDT, J.D. (1972) Collagenolytic enzymes in human neoplasms. Cancer Res.,32. 993-996.

EMERY, P.W., LOVELL, L. and RENNIE, M.J. (1984) Protein synthesis measured in vivo in muscle and liver of cachectic tumour bearing mice. Cancer Res., 44, 2779-2784.

FEARON, K.C.H., BORLAND, W., PRESTON, T., TISDALE, M.J., SHENKIN, A. and CALMAN, K.C. (1988) Cancer cachexia : influence of systemic ketosis on substrate levels and nitrogen metabolism. Am.J.Clin.Nutr., 47, 42-48.

FEARON, K.C.H. and CARTER, D.C. (1985) Cancer cachexia. Br.J.Cancer, 52, 87-92.

FETERIS, W.A. (1965) A serum glucose method without protein precipitation. Am.J.Med.Technol., 31, 17-19.

FISCHER, A. (1946) Mechanism of the proteolytic activity of malignant cells. Nature, 157, 442-446.

FLORES, E.A., BISTRIAN, B.R., POMPOSELLI, J.J., DINARELLO, C.A., BLACKBURN, G.L. and ISTFAN, N.N. (1989) Infusion of tumour necrosis factor/cachectin promotes muscle

catabolism in the rat. A synergistic effect with interleukin-1. J.Clin.Invest., 83, 1614-1622.

FREDERICK, G.L. and BEGG, R.W. (1956) A study of hyperlipidaemia in the tumour-bearing rat. Cancer Res., 16, 548-558.

FREUND, H., HOOVER, H.C., ATAMIAM, S. and FISCHER, J.E. (1979) Infusion of the branched chain amino acids in postoperative patients. Ann.Surg.,190(1), 18-23.

FREUND, H.R., MUGGIA-SULLAM, M., LAFRANCE, R., GALLON, L.S., BARCELLI, U.O. and FISCHER, J.E. (1985) Muscle prostaglandin production in the rat. Effect of abdominal sepsis and different amino acid formulation. Arch.Surg., 120, 1037-1039.

FULKS, R.M., LI, J.B. and GOLDBERG, A.L. (1975) Effects of insulin, glucose and amino acids on protein turnover in rat diaphragm. J.Biol.Chem., 250(1), 290-298.

GARTE, S.J., CURRIE, D.D. and TROLL, W. (1987) Inhibition of H-ras oncogene transformation of NIH3T3 cells by protease inhibitors. Cancer Res., 47, 3159-3162.

GELIN, J., ANDERSSON, C. and LUNDHOLM, K. (1991) Effects of indomethacin, cytokines and cyclosporin A on tumour

growth and the subsequent development of cancer cachexia. Cancer Res., 51, 880-885.

GOLD, J. (1968) Proposed treatment of cancer by inhibition of gluconeogenesis. Oncology, 22, 185-207.

GOLD, J. (1978) Potentiation by Clofibrate of in vivo tumour inhibition by hydrazine sulphate and cytotoxic agents in Walker 256 carcinosarcoma. Cancer Biochem.Biophys., 3, 41-45.

GOLDBERG, A.L. and TISCHLER, M.E. (1981) Regulatory effects of leucine on carbohydrate metabolism. In Walsner, M. and Williamson, J.R. (eds) Metabolism and clinical implications of branched chain amino acids and keto acids p205-215. New York:Elsevier.

GOLDBERG, A.L., BARACOS, V., RODEMANN, P., WAXMAN, L. and DINARELLO, C. (1984) Control of protein degradation in muscle by prostaglandins, Ca^{2+}, and leukocytic pyrogen (interleukin 1). Federation Proc., 43(5), 1301-1306.

GOLL, D.E., OKITANI, A., DAYTON, W.R. AND REVILLE, W.J. (1978) Protein turnover and lysosomal function. Academic press. New York. pp.587-590.

GOODLAD, G.A.J. and CLARK, C.M. (1972) Activity of the

gastrocnemius and soleus polyribosomes in rats bearing the Walker 256 carcinoma. Europ.J.Cancer, 8, 647-651.

GROSSMAN, M.I., CUMMINS, G.M. and IVY, A.C. (1947) The effects of insulin on food intake after vagotomy and sympathectomy. Am.J.Phys., 149, 100-102.

GROUNDWATER, P., BECK, S.A., BARTON, C., ADAMSON, A., FERRIER, I.N. and TISDALE, M.J. (1990) Alteration of serum and urinary lipolytic activity with weight loss in cachectic cancer patients. Br.J.Cancer, 62, 816-821.

HARNET, W.L. (1952) A survey of cancer in London. British Empire Cancer Campaign, London. pp.26.

HART, D.A. and REHEMTULLA, A. (1988) Plasminogen activators and their inhibitors : regulators of extracellular proteolysis. Comp.Biochem.Physiol., 90B, 691-708.

HASSELGREN, P.O., JAGENBURG, R., KARLSTROM, L. PEDERSEN, P. and SEEMAN, T. (1984) Changes of protein metabolism in liver and skeletal muscle following trauma complicated by sepsis. J.Trauma, 24(3), 224-228.

HENSHAW, E.C., HIRSCH, C.A., MORTON, B.E. and HIATT, H.H. (1971) Control of protein synthesis in mammalian

tissues through changes in ribosome activity. J.Biol.Chem., 246(2), 436-446.

HOHORST, H.J. (1974) L-(+)-Lactate. Determination with lactate dehydrogenase and NAD. Methods of enzymatic analysis. 3, pp.1464-1468. Ed. Bergmeyer, H.U.

HOLLAND, J.F. and OHNUMA, T. (1979) Lessons from the study of induced alterations in amino acids in patients with cancer. Cancer Treat.Rep., 65, 55-58.

HOLROYDE, C.P., GABUZDA, T.G., PUTNAM, R.C., PONUL, A. and REICHARD, G.A. (1975) Altered glucose metabolism in metastatic carcinoma. Cancer Res., 35, 3710-3714.

HOLROYDE, C.P. and REICHARD, G.A. (1981) Carbohydrate metabolism in cancer cachexia. Cancer Treat.Rep., 65, 55-58.

HUNTER, D.C., WEINTRAUB, M., BLACKBURN, G.L. and BISTRIAN, B.R. (1989) Branched chain amino acids as the protein component of parenteral nutrition in cancer cachexia. Br.J.Surg., 76, 149-153.

HUSBY, G., STRUCKLAND, R., RIGLER, G., PEAKE, G. and WILLIAMS, R. (1977) Direct immunochemical detections of prostaglandin E and cyclic nucleotides in human malignant

tumours. Cancer, 40, 1629-1642.

HYVARINEN, A. and NIKKIKA, E. (1962) Specific determination of blood glucose with o-toluidine. Clin.Chem.Acta, 7, 140-144.

JAIN, R.K., SHAH, S.A. and FINNEY, P.L. (1984) Continuous noninvasive monitoring of pH and temperature in rat Walker 256 carcinoma during normoglycemia and hyperglycemia. J.N.C.I., 73(2), 429-436.

JEEVANANDAM, M., HOROWITZ, G.D., LOWRY, S.F. and BRENNAN, M.F. (1984) Cancer cachexia and protein metabolism. Sat 30 June, 1423-1426.

KAY, J. (1980) Regulation of proteolysis : implications for the control of protein breakdown within the cell. Biochem.Soc.Trans., 8, 415-417.

KAY, J., SIEMANKOWSKI, L.M., SIEMANKOWSKI, R.F., GREWELING, J.A. and GOLL, D.E. (1982) Degradation of myofibrillar proteins by trypsin-like serine proteinases. Biochem.J., 201, 279-285.

KELLY, M.L., TROUP, S.B., LOGAN, V.W. and TERRY, R. (1961) Carcinoma of cecum with primary malabsorption syndrome. Arch.Int.Med., 108, 174-181.

KIEN, C.L. and CAMITTA, B.M. (1983) Increased whole-body turnover in sick children with newly diagnosed leukemia or lymphoma. Cancer Res. 43, 5586-5592.

KINSELLA, A.R. and RADMAN, M. (1980) Inhibition of carcinogen-induced chromosomal aberrations by an anticarcinogenic protease inhibitor. Proc.Natl.Acad.Sci.USA., 77, 3544-3547.

KISNER, D., HAROSH, M., BLECHER, M., HALLER, D., JACOBS, E., PETERSON, B. and SCHEIN, P. (1978) Malignant cachexia: Insulin resistance and insulin receptors. Proc.Am.Assoc.Cancer Res., 19, 199-208.

KITADA, S., HAYS, E.F., MEAD, J.F. and ZABIN, I. (1982) lipolysis induction in adipocytes by a protein from tumour cells. J.Cell.Biochem., 20, 409-416.

KNOX, L.S. (1983) Nutrition and cancer. Nursing clinics of North America, 18, 97-109.

KRAUSE, R., JAMES, J.H., HUMPHREY, C. and FISCHER, J.E. (1979) Plasma and brain amino acids in Walker 256 carcinoma-bearing rats. Cancer Res., 39, 3065-3069.

KUEHN, L., DAHLMANN, B., HEATH, R. and KAY, J. (1988)

Changes in proteinase/proteinase inhibitor levels in rat skeletal muscle tissue during diabetes and fasting. Biol.J.Chem.Hoppe-Seyler, 369, s299-305.

KUETTNER, K.E., SOBLE, L., CROXEN, R.L., MARCZYNSKA, B., HITI, J. and HARPER, E. (1977) Tumour cell collagenase and its inhibition by a cartilage derived protease inhibitor. Science, 198, 653-654.

LAWSON, D.H., RICHMOND, A., NIXON, D.W. and RUDMAN, D. (1982) Metabolic approaches to cancer cachexia. Ann.Rev.Nutr., 2, 227-301.

LAZO, P.A. (1981) Tumour induction of leucine starvation. Febs.Letters, 135(2), 229-231.

LAZO, P.A. and SOLS, A. (1980) Energetics of tumour cells : enzymic basis of aerobic glycolysis. Biochemical Society Transactions, 8(5), 579.

LEVIN, L., GEVERS, W., JARDINE, L., DE GUELS, F.J.M. and DUNCAN, E.J. (1983) Serum amino acids in weight-losing patients with cancer and tuberculosis. Eur.J.Clin.Oncol., 19(6), 711-715.

LEVINE, L. (1981) Arachidonic acid transformation and tumour promotion. Adv.Cancer Res., 35, 49-79.

LEVINE, L. and WORTH, N. (1984) Eicosapentaenoic acid: Its effects on arachidonic acid metabolism by cells in culture. J.Allergy Clin.Immunol., 74, 430-436.

LEYTUS, S.P., MELHADO, L.L. and MANGEL, W.F. (1983) Rhodamine-based compounds as fluorogenic substrates for serine proteinases. Biochem. J., 209, 299-307.

LIEBELT, R.A., LIEBELT, A.G. and JOHNSTON, H.M. (1971) Lipid mobilisation and food intake in experimentally obese mice bearing transplanted tumours (35924). Proc.Soc.Exp.Biol.Med., 138, 482-490.

LINDSEY, A.M. (1986) Cancer cachexia: Effects of the disease and its treatment. Seminars in Oncology nursing, 2, 19-29.

LIU, H.Y., PELTZ, G.A., LEYTUS, S.P., LIVINGSTON, C., BROCKLEHURST, J. and MANGEL, W.F. (1980) Sensitive assay for plasminogen activator of transformed cells. Proc.Natl.Acad.Sci.USA., 77(7), 3796-3800.

LOWELL, B.B., RUDERMAN, N.B. and GOODMAN, M.N. (1986) Evidence that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal muscle. Biochem.J., 234, 237-240.

LUNDHOLM, K., EDSTROM, S., EKMAN, L., KARLBERG, I., BYLUND, A.C. and SCHERSTEN, T. (1978) A comparative study of the influence of malignant tumour on host metabolism in mice and man. Cancer, 42, 453-461.

LUNDHOLM, K., EDSTROM, S., KARLBERG, I., EHMAN, L. and SCHERSTEN, T. (1980) Relationship of food intake, body composition and tumour growth to host metabolism in non growing mice with sarcoma. Cancer Res., 40, 2515-2522.

LUNDHOLM, K., EDSTROM, S., KARLBERG, I., EHMAN, L. and SCHERSTEN, T. (1982) Glucose turnover, gluconeogenesis from glycerol and estimation of net glucose cycling in cancer patients. Cancer, 42, 453-461.

MAGEE, B.A., POTEZNY, N., ROFE, A.M. and CONYERS, R.A.J. (1979) The inhibition of malignant cell growth by ketone bodies. Australian J.Exp.Biol. and Med.Sci., 57, 529-539.

MAHONY, S.M. and TISDALE, M.J. (1988) Induction of weight loss and metabolic alterations by human recombinant tumour necrosis factor. Br.J.Cancer, 58, 345-349.

MASUNO, H., YAMASAKI, N. and OKUDA, H. (1981) Purification and characterisation of lipolytic factor (toxohormone-L) from cell-free fluid of ascites sarcoma 180. Cancer Res.,

41, 284-289.

MIDER, G.B. (1951) Some aspects of nitrogen and energy metabolism in cancerous subjects : A review. Cancer Res., 11, 821-829.

MILLWARD, D.J. (1980) Protein degradation in muscle and liver. In : Comprehensive Biochemistry. Vol.19B. Elsevier Scientific Publishing Company. pp.153-230.

MOLDAWER, L.L., SVANINGER, G., GELIN, J. and LUNDHOLM, K.G. (1987) Interleukin-1 and tumour necrosis factor do not regulate protein balance in skeletal muscle. Am.J.Phys., 253, C766-770.

NAGY, B., BAN, J. and BRDAR, B. (1977) Production of plasminogen activator by human tumours. Int.J.Cancer., 19, 614-620.

NIXON, D.W., HEYMSFIELD, S.B., COHEN, A.E. (1980) Protein calorie undernutrition in hospitalized cancer patients. Am.J.Med., 68, 683-690.

NIXON, D.W., LAWSON, D.H., KUTNER, M., ANSLEY, J., SCHWARZ, M., HEYMSFIELD, S., CHAWLA, R., CARTWRIGHT, T.H. and RUDMAN, D. (1981) Hyperalimentation of the cancer patient with protein calorie undernutrition. Cancer Res.,

41, 2038-2045.

NORTON, J.A., MOLEY, J.F., GREEN, M.V., CARSON, R.E., and MORRISON, S.D. (1985) Parabiotic transfer of cancer anorexia/cachexia in male rats. Cancer Res., 45, 5547-5552.

OLIFF, A. (1988) The role of tumour necrosis factor (cachectin) in cachexia. Cell, 54, 141-145.

OSSOWSKI, L. and REICH, E. (1983) Antibodies to plasminogen activator inhibit human tumour metastasis. Cell, 35, 611-619.

OTTOSON, R. and SYLVEN, B. (1960) Changes in the dipeptidase and acid proteinase activities in blood plasma of mice carrying ascites tumours. Arch.Biochem.Biophys., 87, 41-47.

PAIN, V.M. and CLEMENS, M.J. (1980) Protein synthesis in mammalian systems. In : Comprehensive Biochemistry. Vol.19B, Elsevier Scientific Publishing Company. pp.1-56.

PALMER, R.M. and WAHLE, K.W.J. (1987) Protein synthesis and degradation in isolated muscle - effect of omega-3 and omega-6 fatty acids. Biochem.J., 242, 615-618.

PEDERSON, P.L. (1978) Tumour mitochondria and the bioenergetics of cancer cells. In : Progress in experimental tumour research. Vol.22, Karger, Basel. pp.190-274.

PERRY, J.K. and SCOTT, G.K. (1990) Cell surface and secreted proteinases and guanidinobenzoatase activity. Bio.Sci.Rep., 10(5), 469-472.

QUIGLEY, J.P. (1979) Proteolytic enzymes of normal and malignant cells. Haynes, R. Chichester, Wiley. pp 247-285.

QUIGLEY, J.P., OSSOWSKI, L. and REICH, E. (1974) Plasminogen, the serum proenzyme activated by factors from cells transformed by oncogenic viruses. J.Biol.Chem., 249, 4306-4311.

RANG, H.P. and DALE, M.M. (1988) Pharmacology. Churchill Livingstone, Edinburgh. 189-198.

RECKLIES, A.D., TILTMAN, K.J., STOKER, T.A.M. and POOLE, A.R. (1980) Secretion of proteinases from malignant and non malignant human breast tissue. Cancer Res., 40, 550-556.

REDGRAVE, T.G., DEVEREUX, D.F. and DECKERS, P.J. (1984) Hyperlipidemia in tumour-bearing rats. Biochim et

Biophys.Acta, 795, 286-292.

REICH, R., ROYCE, L. and MARTIN, G.R. (1989) Eicosapentaenoic acid reduces the invasive and metastatic activities of malignant tumour cells. Biochem.Biophys.Res.Comm., 160(2), 559-564.

REICH, R., THOMPSON, E.W., IWAMOTO, Y., MARTIN, G.R., DEASON, J.R., FULLER, G.C. and MISKIN, R. (1988) Effects of inhibitors of plasminogen activator, serine proteases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res., 48, 3307-3312.

RENNIE, M.J., EDWARDS, R.H.T., EMERY, P.W., HALLIDAY, D., LUNDHOLM, K. and MILLWARD, D.J. (1983) Depressed protein synthesis is the dominant characteristic of muscle wasting and cachexia. Clin.Phys., 3, 387-398.

RIFKIN, D.B. and CROWE, R.M. (1977) Isolation of a protease inhibitor from tissues resistant to tumour invasion. Hoppe-Seyler's Z. Physiol. Chem.,358, 1525s-1531s.

RIVERA, S., AZCON-BIETO, J., LOPEZ-SORIANO, F.J., MIRALPEIX, M. and ARGILES, J.M. (1988) Amino acid metabolism in tumour-bearing mice. Biochem.J., 249, 443-449.

ROBLIN, R. (1978) Contributions of secreted tumour cell products to metastasis. Cancer Biol. Rev., 2, 59-93.

RODEMANN, H.P. and GOLDBERG, A.L. (1982) Arachidonic acid, prostaglandin E_2 and $F_{2\alpha}$ influence rates of protein turnover in skeletal and cardiac muscle. J.Biol.Chem., 257(4), 1632-1638.

ROH, M.S., EKMAN, L., JEEVANANDAM, M. and BRENNAN, M.F. (1984) Gluconeogenesis in tumour influenced hepatocytes. Surgery, 427-434.

ROITT, I., BROSTOFF, J. and MALE, D. (1988) Immunology. Churchill Livingstone. 18.1-18.15.

RONDEAU, E., ANGLES-CANO, E., DELARUE, F., SULTAN, Y. and SRAER, J.D. (1986) Polyunsaturated fatty acids increase fibrinolytic activity of human isolated glomeruli. Kidney Int., 30, 701-705.

ROTMAN, E.I., BROSTROM, M.A. and BROSTROM, C.O. (1992) Inhibition of protein synthesis in intact mammalian cells by arachidonic acid. Biochem.J., 282, 487-494.

ROUGHLEY, P.J., MURPHY, G. and BARRETT, A.J. (1978) Proteinase inhibitors of bovine nasal cartilage. Biochem.

J., 169, 721-724.

SAMESHIMA, M., LIEBHABER, S.A. and SCHLESSINGER, D. (1981) Dual pathways for RNA turnover in WI-38 but not in I-cell human diploid fibroblasts. Mol.Cell.Biol., 1, 75-81.

SCAMBIA, G., BENEDETTI, P.P., FERRANDINA, G., BATTAGLIA, F., ROSSI, S., BELLATONE, R., CRUTTI, F. and MANCUSO, S. (1991) Cathepsin D and epidermal growth factor in breast cyst fluid. Br. J. Cancer, 64, 965-967.

SCHAUR, R.J., SEMMELROCK, H.J., SCHREIBMAYER, W., TILLIAN, H.M. and SCHAUENSTEIN, T. (1980) Tumour host relations. V. Nitrogen metabolism in Yoshida sarcoma bearing rats, reduction of growth rate and increase of survival time by administration of physiological doses of branched chain amino acids. J.Cancer Res.Clin.Oncol., 97, 285-293.

SCHEIN, P.S., KISNER, D., HALLER, D., BLECHER, M. AND HAMOSH, M. (1979) Cachexia of malignancy. Potential role of insulin in nutritional management. Cancer, 43, 2070-2076.

SCHEIN, P.S., MACDONALD, J.S., WATERS, C. and HAIDAK, D. (1975) Nutritional complications of cancer and its treatment. Seminars in Oncology, 2(4), 337-347.

SCHELP, F.P. and PONGPAEW, P. (1988) Protection against cancer through nutritionally-induced increase of endogenous proteinase inhibitors - a hypothesis. Int.J.Epidemiol., 17, 287-292.

SHAMBERGER, R.J. (1984) Cancer cachexia. In: Nutrition and cancer. Plenum press, London. 353-366.

SHAPOT, V.S. and BLINOV, V.A. (1974) Blood glucose levels and gluconeogenesis in animals bearing transplantable tumours. Cancer Res., 34, 1827-1832.

SHERWIN, R.S., HENDLER, R.G. and FELIG, P. (1975) Effect of ketone infusion on amino acid and nitrogen metabolism in man. J.Clin.Invest., 15, 1382-1390.

SHONHEYDER, F., HEILSKOV, N.C.S. and OLESON, K. (1954) Isotopic studies on the mechanism of negative nitrogen balance produced by immobilization. Scand.J.Clin.Lab.Invest., 6, 178-188.

STEIN, T.P. (1978) Cachexia, gluconeogenesis and progressive weight loss in cancer patients. J.Theor.Biol., 73, 51-59.

STEVEN, F.S., ALI, H. and GRIFFIN, M.M. (1988d) The

inhibition of a tumour cell surface protease in vivo and its reactivation by oxidation. Br.J.Cancer, 57, 160-164.

STEVEN, F.S. and AL-AHMED, R.K. (1983) Evidence for an enzyme which cleaves the guanidinobenzoate moiety from active site titrants specifically designed to inhibit and quantify trypsin. Eur.J.Biochem., 130, 335-339.

STEVEN, F.S. and BOOTH, N.A. (1991) The inhibitor reacting with a tumour cell surface protease can be exchanged with plasminogen activator inhibitor (PAI-1). J.Enz.Inhib., 4(3), 273-279.

STEVEN, F.S. and BLAKEY, D.C. (1992) The role of fibrin fibrils in the dissociation of a cell surface protease - inhibitor complex and evidence for the recapture of the inhibitor protein. J.Enz.Inhib., 5, 299-315.

STEVEN, F.S., GRIFFIN, M.M., CEDERHOLM-WILLIAMS, S.A., MANGEL, W.F. and MAIER, H. (1991) Evidence for the functional similarity between tumour cell surface guanidinobenzoatase and tissue-type plasminogen activator. Anticancer Res., 11, 641-648.

STEVEN, F.S. and GRIFFIN, M.M. (1988c) Inhibitors of guanidinobenzoatase and their possible role in cell migration. Biol.Chem.Hoppe-Seyler, 369, 137s-143s.

STEVEN, F.S. and GRIFFIN, M.M. (1990) Similarities between single-chain plasminogen activator and the cell surface proteinase, guanidinobenzoatase. Biochem. Soc. Trans., 18(4), 632-633.

STEVEN, F.S., GRIFFIN, M.M., FREEMONT, A.J. and JOHNSON, J. (1988f) Inhibition of guanidinobenzoatase: evidence for multiple forms of this protease on different tumour cells. J.Enz.Inhib., 2, 117-127.

STEVEN, F.S., GRIFFIN, M.M., WONG, T.L.H. and ITZHAKI, S. (1986) Evidence for inhibitors of the cell surface protease guanidinobenzoatase. J.Enzyme Inhib., 1, 127-137.

STEVEN, F.S., GRIFFIN, M.M., MANGEL, W.F., MAIER, H. and ALTMANNSBERGER, M. (1988a) Inhibition of guanidinobenzoatase by a substrate for trypsin-like enzymes. J.Enzyme Inhib., 2, 209-214.

STEVEN, F.S., GRIFFIN, M.M. and AL-AHMAD, R.K. (1985) The design of fluorescent probes which bind to the active centre of guanidinobenzoatase. Eur.J.Biochem., 149, 35-40.

STEVEN, F.S., GRIFFIN, M.M., ITZHAKI, S. and Al-HABIB, A. (1981) Inhibition properties of sepharose bound trypsin and a protease on the surface of Ehrlich ascites tumour cells.

Biochim. et Biopohys. acta, 660, 333-340.

STEVEN, F.S., GRIFFIN, M.M., MAIER, H., WEIDAUER, H., MANGEL, W.F. and ALTMANNSBERGER, M. (1988b) Studies on the activity of a protease associated with cells at the advancing edge of human tumour masses in frozen sections. Br.J.Cancer, 58, 56-60.

STEVEN, F.S., GRIFFIN, M.M., WILLIAMS, L.A., ALI, H. and MAIER, H. (1989) Targeting adriamycin to tumour cells by means of an affinity ligand; a model system for drug delivery. Anticancer Res., 9, 247-253.

STEVEN, F.S. and HILL, R.J. (1988e) A study of guanidinobenzoatase during development of mesothelioma induced in the rat by fibrous erionite. Br.J.Cancer, 58, 610-613.

STEVEN, F.S., MAIER, H., ARNDT, J. and BORN, I.A. (1990) Evidence for the induction of protease activity on cultured tumour cells as a consequence of implantation into nude mice. Cancer Lett., 50, 191-196.

STEVEN, F.S., SURESH, U., WONG, T.L.H. and GRIFFIN, M.M. (1987) The role of inhibitors in the fluorescent staining of benign naevus and malignant melanoma cells with 9-amino acridine orange. J.Enzyme Inhib., 1, 275-287.

STOVROFF, M.C., FRAKER, D.C., SWEDENBORG, J.A. and NORTON, J.A. (1988) Cachectin / Tumour necrosis factor. A possible mediator of cancer anorexia in the rat. Cancer Res., 48, 4567-4572.

STRELKOV, A.B., FIELDS, A.L.A. and BARACOS, V. (1989) Effect of systemic inhibition of prostaglandin production on protein metabolism in tumour-bearing rats. Am.J.Phys., 257, C261-269.

STRYER, L. (1981) Biochemistry : Muscle contraction and cell motility. pp.816-827. W.H. Freeman and Company.

TASHJIAN, A., VOELKEL, F., GOLDHABER, P. and LEVINE, L. (1974) Prostaglandins, calcium metabolism and cancer. Federation Proc., 33, 81-86.

TATESON, J.E., RANDALL, R.W., Reynolds, C.H., JACKSON, W.P., BHATTACHERJEO, P., SALMON, J.A. and GARLAND, L.G. (1988) Selective inhibition of arachidonate 5-lipoxygenase by novel acethydroxamic acids : biochemical assessment in vitro and ex vivo. Br.J.Pharmacol., 94, 528-539.

TESSITORE, L., BONELLI, G. and BACCINO, F.M. (1987) Early development of protein metabolic perturbations in the liver and skeletal muscle of tumour bearing rats. Biochem.J.,

241, 153-159.

TEREPKA, A.R. and WATERHOUSE, C. (1956) Metabolic observations during the forced feeding of patients with cancer. Am.J.Med., 20, 225-238.

THEOLOGIDES, A. (1972) Pathogenesis of cachexia in cancer : a review and a hypothesis. Cancer, 29, 484-488.

THEOLOGIDES,A. (1976) Anorexia-producing intermediary metabolites. Am.J.Clin.Nutr., 29, 552-558.

THOMSON, M., KOONS, J., TAN, E.T.H. and GRIGOR, M.R. (1981) Modified lipoprotein lipase activities, rates of lipogenesis and lipolysis as factors leading to lipid depletion in C57BL6 mice bearing preputial gland tumour ESR 586. Cancer Res., 41, 3228-3232.

THORPE, S.M., ROCHEFORTE, H., GARCIA, M., FREISS, G., CHRISTENSEN, I.J., KHALAF, S., PAOLUCCI, F., PAU, B., RASMUSSEN, B.B. and ROSE, C. (1989) Association between high concentrations of Mr 52,000 cathepsin D and poor prognosis in primary human breast cancer. Cancer Res., 49, 6008-6014.

TISCHLER, M.E., DESAUTELS, M. and GOLDBERG, A.L. (1982) Does leucine, leucyl-tRNA, or some metabolite of leucine

regulate protein synthesis and degradation in skeletal cardiac muscle. J.Biol.Chem., 257(4), 1613-1621.

TISDALE, M.J. (1982) Tumour and host nutrition. Cancer Topics, 3, 113-117.

TISDALE, M.J. (1986) The future : Nutritional pharmacology. Clinics in Oncology, 5(2), 381-405.

TISDALE, M.J. (1990) Newly identified factors that alter host metabolism in cancer cachexia. Trends in Pharmacological Science, 11, 473-475.

TISDALE, M.J., BECK, S.A. (1991) Inhibition of tumour induced lipolysis in vitro and cachexia and tumour growth in vivo by eicosapentaenoic acid. Biochem.Pharmacol., 41, 103-109.

TISDALE, M.J. and BRENNAN, R.A. (1983) Loss of acetoacetate coenzyme A transferase activity in tumours of peripheral tissues. Br.J.Cancer, 47, 293-297.

TROLL, W., WEISNER, R. and FRENKEL, K. (1987) Anticarcinogenic action of protease inhibitors. Adv. in Cancer Res., 49, 265-283.

UNKELESS, J.C., DANO, K., KELLERMAN, G.M. and REICH, E. (1974) Fibrinolysis associated with oncogenic

transformation. J.Biol.Chem., 249, 4295-4305.

VAN VENROOIJ, W.J.W., HENSHAW, E.C. and HIRSCH, C.A. (1972) Effects of deprival of glucose or individual amino acids on the polyribosome distribution and rate of protein synthesis in cultured mammalian cells. Biochim,Biophys.Acta, 259, 127-137.

VASSALLI, J.D., HAMILTON, J. and REICH, E. (1976) Macrophage plasminogen activator : modulation of enzyme production by anti-inflammatory steriods, mitotic inhibitors, and cyclic nucleotides. Cell, 8, 271-281.

VERHEIJEN, J.H., CHANG, G.T.G. and KLUFT, C. (1984) Evidence for the occurence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. Thromb.Haemostas., 51(3), 392-395.

VERLOES, R., ATASSI, G., DUMONT, P. and KANAREK, L. (1978) Tumour growth inhibition mediated by trypsin inhibitor or urokinase inhibitors. Europ.J.Cancer., 14, 23-31.

WAAKES, T.P. and UDENFRIEND, S. (1957) A fluorimetric method for the estimation of tyrosine in plasma and tissues. J.Lab.Clin.Med., 50(5), 733-736.

WANG, B.S., McLOUGHLIN, G.A., RICHIE, J.P. and MANNICK,

J.A. (1980) Correlation of the production of plasminogen activator with tumour metastasis in B16 mouse melanoma cell lines. Cancer Res., 40, 288-292.

WARBURG, O. (1930) Metabolism of tumours. Arnold Constable. London.

WARNER, B.W., HASSELGREN, P.O., HUMMEL, R.P., JAMES, J.H., PEDERSEN, P. and FISCHER, J.E. (1990) Effect of catabolic hormone infusion on protein turnover and amino acid uptake in skeletal muscle. Am.J.Surg., 159, 295-300.

WARREN, S. (1932) The immediate cause of death in cancer. Am.J.Med., 184, 610-616.

WATERHOUSE, C., JEANPETRE, N. and KEILSON, J. (1979) Gluconeogenesis from alanine in patients with progressive malignant disease. Cancer Res., 39, 1968-1972.

WATERHOUSE, C., KEMPERMAN, J.H. and STORMONT, J.M. (1964) Alterations in triglyceride metabolism as produced by dietary change. J.lab.Clin.Med., 63, 605-620.

WESDORP, R.I.C. (1986) Role of abnormal metabolism in the aetiology of cancer cachexia. Clinics in Oncology, 5(2), 307-315.

WHITE, D.A., MIDDLETON, B. and BAXTER, M. (1984) Hormones and metabolic control. Arnold Press.

WILCOX, J.C., CORR, J., SHAW, J., RICHARDSON, M and CALMAN, K.C. (1984) Prednisolone as an appetite stimulant in patients with cancer. Br.Med.J., 288, 27-32.

WILLIAMS, J.F. and MATTHAEI, K.I. (1981) Cancer induced body wasting - a review of cancer cachexia and a hypothesis concerning the molecular basis of the condition. Asean J.Clin.Sci., 2(2), 158-167.

WISEMAN, G. and GHADIALLY, F.N. (1955) Studies in amino acid uptake by RD3 sarcoma cell suspensions in vitro. Br.J.Cancer, 9(3), 480-485.

YAVELOW, J., FINLAY, T.H., KENNEDY, A.R. and TROLL, W. (1983) Bowman-Birk soybean protease inhibitor as an anticarcinogen. Cancer Res., 43, 2454s-2459s.

YOUNG, R. and NEWBY, M. (1986) Enhancement of Lewis lung carcinoma cell migration by prostaglandins E_2 produced by macrophages. Cancer Res., 46, 160-164.

APPENDIX.

List of publications.

Abstract.

SMITH, K.L., BECK, S.A. and TISDALE, M.J. (1992) Protein turnover in skeletal muscle in cancer cachexia. 33rd Annual meeting of the British Association for Cancer Research and the 7th Annual meeting of the Association of Cancer Physians. University of Southampton, UK. 29th March - 1st April.

Paper.

BECK, S.A., SMITH, K.L. and TISDALE, M.J. (1991) Anticachectic and antitumour effect of EPA and its effect on protein turnover. Cancer Res., 51, 6089-6093.

Aston University

An in vitro method of measuring protein synthesis and degradation was used primarily because protein degradation had previously (Beck and Tisdale, 1987) been shown to be an important characteristic of cachexia associated with the MAC16 tumour. Further studies comfirmed that these observations were associated with a circulating proteolytic factor. Thus subsequent measurements were directed toward elucidating a mediator of increased protein degradation rather than actually quantifying absolute rates of protein turnover through an in vivo method. The in vitro method of measuring protein degradation descibed essentially by Wu and Thompson, (1990) minimised the variables associated with a biological system and enabled a rapid, reproducible and efficient use of animals.

One minor criticism of the method used to measure protein synthesis is that no measurement of the intracellular free unlabelled pool of phenylalanine was made - consequently changes in the plasma amino acid concentration would affect intracellular pools with an effect on labelled amino acid uptake and incorporation. However, intracellular and extracellular concentrations of labelled phenylalanine have been shown to equilibrate within 2 min of administration and the intracellular concentration is used as a parameter in the calculation of protein synthesis.

The limitations and problems associated with a direct

comparison of in vitro and in vivo methods is highlighted in Fig.13. Protein degradation is indicated to be massively increased up to 30% weight loss - suggesting a chronic depletion of gastrocnemius muscle and host body nitrogen content. However, this is not evident from the graphs shown in Fig.11 and 12. The in vitro measurement of protein degradation may be exagerated by the susceptibility of this muscle group to the cachectic effect of the tumour (section 4.03) or it may be attributable to an artifact associated with progressive changes in the size of the muscles intracellular free phenylalanine pool. The latter reason is unlikely as the free phenylalanine pool is not a participant in the calculation for protein degradation.

Inconsistencies may also be encountered when comparing results obtained from differing methodologies even though comparable principles are involved. For example the tritiated phenylalanine method showed that the highest rates of skeletal muscle proteolysis were observed at the time of greatest weight loss (Fig.13). However, serum from cachectic MAC16 tumour-bearing animals with 21-25% weight loss did not elicit a comparable degree of degradation when measured using tyrosine release (Fig.17). This is an unfair comparison as a number of important parameters are unequal. The experiments were designed to demonstrate a particular facet of muscle proteolysis. The release of tritiated phenylalanine from preloaded muscles from animals

with progressive weight loss illustrates that protein degradation becomes increasingly important. This is a reflection of events at the muscle level with no external stimuli. This contrasts to the tyrosine release measured from non cachectic control muscle under the influence of serum from animals with progressive weight loss. This experiment was designed to demonstrate the effects of differing levels of a proteolytic factor on a fixed entity i.e. control muscle. The combination of the two experiments demonstrates that at high weight losses the muscle of cachectic animals is already "switched on" to a high rate of degradation and that the stimulus for this may have been the circulating factor evident most prominently at 11-15% weight loss.

The limited amount of serum obtained by cardiac puncture from animals with 11-15% weight loss necessitated the pooling of serum. This enabled a direct comparison of results from a particular batch of serum (Fig.50). There are however variations between batches of serum (see Fig.54, 55). Where comparisons are made between batches it is always necessary to apply judicious controls to ensure reproducibility.