INHIBITORS OF LIPOLYSIS IN TUMOUR-INDUCED CACHEXIA

CAROLYN LIESMA ADAMSON

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THE UNIVERSITY OF ASTON IN BIRMINGHAM

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Inhibitors of Lipolysis in Tumour-Induced Cachexia

Carolyn Liesma Adamson

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SUMMARY

The MAC16 tumour produces a factor which exhibits lipid-mobilizing activity in vitro in addition to causing extensive depletion of host lipid stores. The mechanism of the anti-lipolytic effect of two anti-cachectic agents, eicosapentaenoic acid, an ω-3 polyunsaturated fatty acid (PUFA), and N-(3-phenoxycinnamyl)acetohydroxamic acid (BW A4C), a 5-lipoxygenase inhibitor, has been investigated. These two agents reduce tumour growth and reverse the weight loss which accompanies transplantation of the MAC16 murine colon adenocarcinoma into NMRI mice.

Mice transplanted with the MAC16 tumour exhibited weight loss which was directly proportional to the serum lipolytic activity measured in vitro up to a weight loss corresponding to 16% of the original body weight. After this time, an inverse relationship between weight loss and lipolytic activity was observed. Body composition analysis revealed a large decrease in body fat relative to other body compartments.

The anti-tumour/anti-cachectic effect of EPA did not appear to be due to its ability to inhibit the production of prostaglandin E_2.

The MAC16 lipolytic factor increased adenylate cyclase activity in adipocyte plasma membranes in a concentration-dependent manner. EPA inhibited the production of cAMP attributed to this lipid-mobilizing factor.

EPA produced alterations in G_i, the guanine nucleotide - binding protein which mediates hormonal inhibition of adenylate cyclase, in addition to altering cAMP production in adipocyte plasma membranes in response to hormonal stimulation. The alterations in adenylate cyclase activity were complex and not specific to EPA. EPA stimulated adenylate cyclase activity when in a relatively high fatty acid : membrane ratio and inhibited activity when this ratio was lowered. The inhibitory effect of EPA on adenylate cyclase activity may be the underlying mechanism which explains its anti-lipolytic and anti-cachectic effect. The inability of the related ω-3 PUFA, docosahexaenoic acid (DHA), to inhibit cachexia may be due to a difference in the metabolic fates of these two fatty acids.

BW A4C inhibited lipolysis in isolated adipocytes which suggests that this compound may possess the potential for an anti-cachectic effect which is independent of its inhibitory effect on tumour growth.

KEY WORDS: ADENYLATE CYCLASE, BW A4C, CANCER CACHEXIA, G-PROTEINS, EICOSAPENTAENOIC ACID, LIPOLYTIC FACTOR.

To Mum, with love

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ABBREVIATIONS

AA	Arachidonic acid
AC	Adenylate cyclase
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
cAMP	Cyclic adenosine 5',3'-monophosphate
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol - bis (β-aminoethyl ether) N,N,N',N'- tetraacetic acid
EtOH	Ethanol
F	Fluorescence
FAD	Flavine adenine dinucleotide
$FADH_2$	Flavine adenine dinucleotide, reduced form
FCS	Foetal calf serum
fMLP f-Met-Leu-Phe	Formylmethionylleucylphenylalanine
G	G-protein
GLA	Gamma linolenic acid
GMP	Guanosine 5'-monophosphate
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
H	Hormone
HEPES	N-2-Hydroxyethylpiperazine-N-2-

	ethanesulphonic acid
HETE	5-Hydroxy-6,8,11,14-eicosatetraenoic acid
Iso	Isoprenaline
K_{act}	Activation constant
K_d	Dissociation constant
LA	Linoleic acid
LT	Leukotriene
MCT	Medium chain triglyceride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NBT	Nitrobluetetrazolium
p	Probability
PG	Prostaglandin
PKC	Protein kinase C
PT	Pertussis toxin
PUFA	Polyunsaturated fatty acid
Quin 2-AM	Quin 2-acetoxymethyl ester
R	Receptor
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
sp. act.	Specific activity
STT	Students t-test
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	Tumour necrosis factor
TPA	12-O-Tetradecanoylphorbol-13-acetate

TPN	Total parenteral nutrition
Tris	Tris(hydroxymethyl)methylamine
VLDL	Very low density lipoprotein

All other abbreviations refer to SI units.

SECTION 1 : INTRODUCTION

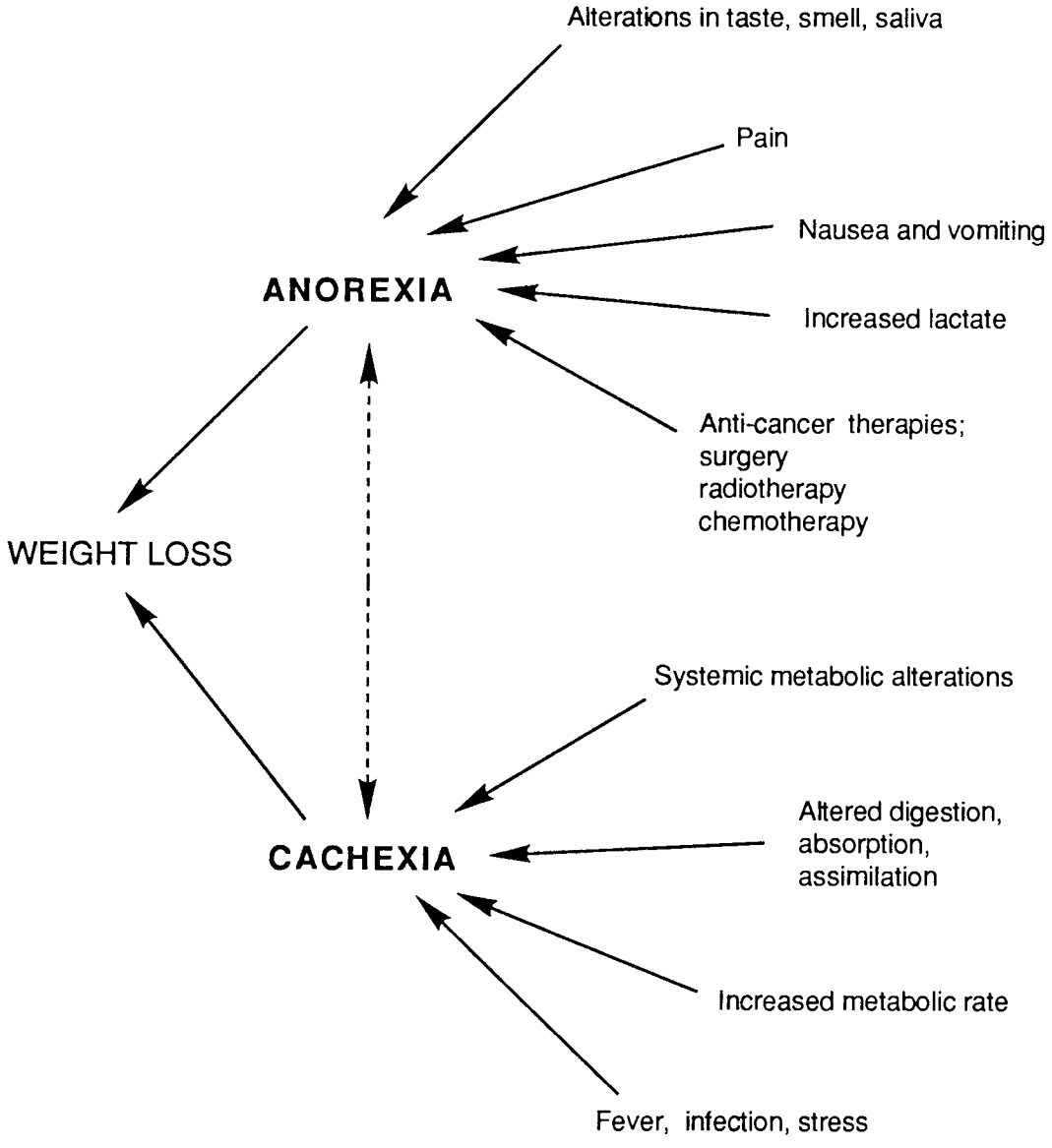
1.0 General Introduction

The word cachexia is derived from the Greek words "kakos hexis", meaning "bad condition" (Shamberger, 1984). Cancer cachexia has been defined as a clinical state in which the principal symptoms are anorexia, early satiety, weight loss, anaemia and marked asthenia (Theologides, 1979). It is commonly the most debilitating feature of malignant disease. Studies have repeatedly shown that the rate and degree of weight loss does not correlate with the histology or site of the tumour, the stage of disease, or the number of metastases present (Schein et al, 1979; Theologides, 1979). Indeed weight loss is often the only presenting symptom of a new malignancy where the tumour is still small whereas some patients with advanced tumours maintain a good nutritional status (Schein et al, 1979).

1.1 The Cachexia-Anorexia Syndrome

Many cancer patients experience what is known as the cachexia-anorexia syndrome (shown in fig. 1.1), which varies in frequency from one tumour to another (Body et al, 1987). The syndrome occurs in 31-40% of patients with favourable subtypes of breast cancer, acute non-lymphocytic leukaemia or non-Hodgkin's lymphoma, and in 83-87% of patients with gastric or pancreatic cancer (DeWys, 1986). The incidence of cachexia-anorexia in the diseases quoted may be due in part to their

Figure 1.1



Factors Influencing Weight Loss in Cancer Cachexia

Lindsey, 1986

location. Non-Hodgkin's lymphoma and tumours of the breast are usually palpable at an early stage of the disease. This leads to prompt diagnosis and reversal of accompanying cachexia with successful treatment of the malignancy. Gastric and pancreatic tumours are likely to cause weight loss due to their sites and also due to the fact that with these tumour sites early symptoms are likely to be non-specific thus leading to a delay in diagnosis. Treatment at a late stage may be ineffective and progression of the disease will lead to the continuation of an accompanying cachectic state. These examples provide an explanation for the expected occurrence of cachexia but do not explain the incidence of severe cachexia where the tumour burden is small.

The three methods of treatment of most cancers (surgery, radiotherapy and chemotherapy) may also lead to weight loss in the patient. Nausea, vomiting and diarrhoea are common side-effects of treatment as well as being a manifestation of the disease itself. Impaired digestion/ malabsorption may also occur as a result of surgery, radiotherapy or chemotherapy.

Mechanical obstruction of the gastro-intestinal tract by the tumour itself in addition to ulceration or infection of the mouth and abnormalities of taste (Carson and Cormicon, 1977) may lead to a decrease in food intake or absorption. Learned food aversions are also a problem in patients undergoing aggressive treatment. Familiar food consumed just prior to chemotherapy may become the object of these aversions

(Bernstein et al, 1982). Cancer patients also tend to exhibit high levels of lactate in their blood. An exogenous supply of lactate has been shown to cause anorexia, nausea and anxiety in normal subjects and therefore carbohydrate-rich foods which lead to an increase in blood lactate levels may produce an aversion to this type of food (DeWys, 1985).

Another problem with cancer patients is that they show evidence of early satiety. Stimulation of appetite via the ventral hypothalamus occurs in response to stimulation of peripheral sensory receptors in the mouth, stomach and intestine and also to low levels of circulating nutrients. The high levels of free fatty acids, amino acids and glucose observed in the blood of some cancer patients may therefore suppress appetite. Theologides (1972) suggested that small molecules secreted by the tumour itself may affect appetite by direct or indirect action on the hypothalamus.

Depression, of which anorexia is a classic symptom, is common in cancer patients. In a study by Derogatis et al (1983) less than 10% of cancer patients had been diagnosed as clinically depressed, although almost 50% had some psychiatric disorders, particularly "adjustment disorders". Transient anorexia linked to pain or emotional distress is common, particularly at the initial diagnosis or recurrence of a malignancy (Holland et al, 1977).

General debility due to the disease state is a factor predisposing to anorexia as well as being a symptom of cancer cachexia. Eating requires energy expenditure and therefore a

patient lacking in energy may not wish to eat thus leading to a viscious circle (DeWys, 1985). Excessive protein loss due to tumour necrosis, ulceration, associated infection or haemorrhage may also lead to loss of body weight (Anonymous, 1978).

Finally, in an attempt to explain the weight loss seen in cancer patients which is not consistent with any of the above explanations, the possibility of specific metabolic alterations induced by the tumour must be considered.

1.2 Metabolic Alterations in Cancer Cachexia

Theologides (1979) suggested that the syndrome of cancer cachexia is due to a more complex metabolic pattern than can be explained by a state of simple starvation. Many cancer patients lose weight with no apparent reduction in food intake (Ashworth, 1984) and with no evidence of malabsorption resulting from infection or a post-operative state. The weight loss is often reversed with successful treatment of the underlying neoplasm (Schein et al, 1979). This observation has also been made in controlled animal experiments such as those utilizing the murine MAC16 colon adenocarcinoma which produces a marked cachexia in the host without a reduction in food and water intake (Bibby et al, 1987). In order to understand the complex metabolic changes induced in the host by the presence of a tumour it is necessary to regard the tumour and its host as two separate entities (Costa, 1963).

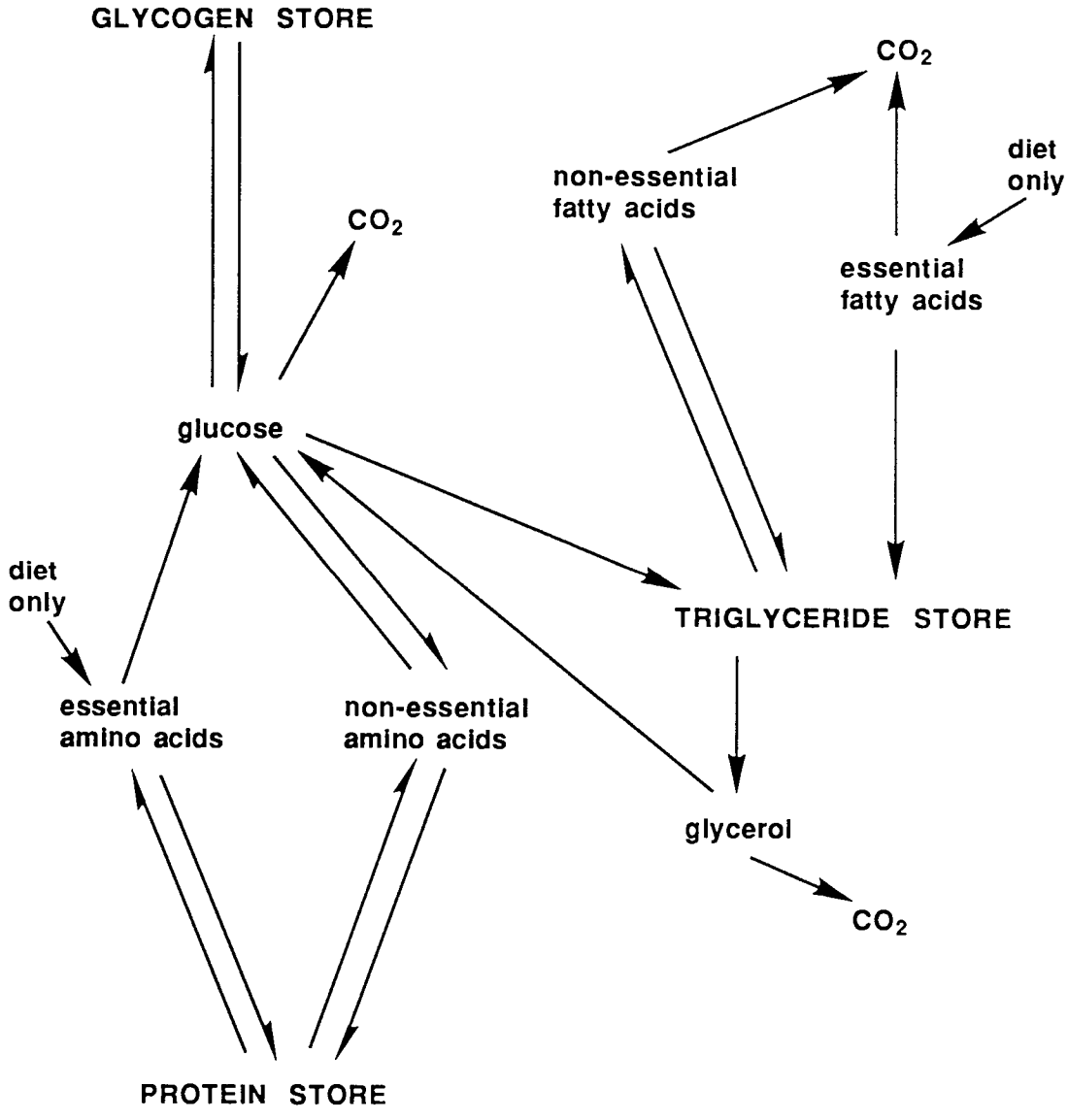
1.2.1 Tumour-Host Metabolic Interactions

The alterations in host metabolism experienced in malignant disease may be extensive, possibly affecting every metabolic process. Alterations in carbohydrate, lipid and protein metabolism (fig. 1.2) have all been noted in addition to fluid and electrolyte imbalance (Shamberger, 1984).

1.2.1.1 Basal Metabolic Rate

In starvation the body adjusts in order to conserve energy. Blood glucose levels are maintained initially in the absence of dietary carbohydrate by rapid proteolysis which provides amino acids for the gluconeogenic pathway. This results in an increased elimination of urinary nitrogen in the form of urea. Glucagon stores are mobilized and the excess of plasma glucagon over insulin leads to mobilization of fatty acids to provide an energy source for peripheral tissues thus reserving limited glucose supplies for the brain. The result is an excess of acetyl CoA from fatty acid breakdown with respect to oxaloacetate from the glycolytic pathway (oxaloacetate is a product of glycolysis and a substrate for gluconeogenesis which are decreased and increased respectively in the starved state). This excess leads to the diversion of acetyl CoA to form ketone bodies. These molecules form an energy source initially for peripheral tissues in order to preserve the brain's glucose supply but in prolonged

Figure 1.2



Interconvertibility of Fat, Carbohydrate and Protein

Adapted from Gurr and James, 1975

starvation, where the glucose levels are extremely low, the brain can adapt to use ketone bodies as a fuel, thus sparing lean body tissue by decreasing the requirement for gluconeogenesis. The presence of ketone bodies leads to conservation of lipid stores by a direct effect and also due to stimulation of insulin secretion. The overall effect is therefore a reduction in basal metabolic rate.

The situation is quite different in the case of "tumour-induced starvation" of the host. An increased basal metabolic rate has been reported in cancer patients (Warnold et al, 1978). In animals the transplantation of a tumour can lead to an almost immediate increase in energy expenditure, before the tumour itself can be palpated (Pratt and Putney, 1958). A study by Bozetti et al (1980) showed an increase in metabolic rate which was proportional to the severity of the weight loss experienced. Another by Knox et al (1983) suggested that patients with the highest metabolic rates were those who had had their malignancy for the longest. A study in which a 10% increase in metabolic rate was seen in patients with metastatic disease showed a poorer correlation between metabolic rate and lean body mass in patients with metastases than in those with localized, or especially no, malignant disease (Macfie et al, 1982). The rise in protective ketone bodies seen in simple starvation is not seen in cancer patients which may, at least in part, account for the prolonged breakdown of adipose and lean body tissue (Tisdale, 1986).

1.2.1.2 Glucose Metabolism

Many tumours display a requirement for glucose to support their growth and an increase in the rate of glycolysis in tumour-bearing hosts is often observed. In the case of large solid tumours with a poor blood supply glucose may be the only source of energy for the tumour since the only mechanism of ATP generation in the absence of oxygen is the Embden-Meyerhoff pathway (Tisdale, 1986).

Increased glycolysis in tumour cells may also occur due to high tumour lactate dehydrogenase activity. Lactate dehydrogenase operates under anaerobic conditions and increased activity would lead to higher circulating levels of lactate which is in agreement with the observation of increased Cori cycle activity in cancer patients (Holroyde et al, 1975). The Cori cycle produces two molecules of ATP from the glycolytic conversion of glucose to lactate in peripheral tissues (or the tumour). Lactate is then converted back to glucose in the liver at the expense of six molecules of ATP. This increase in gluconeogenesis from lactate in a futile attempt to restore glucose homeostasis in the host leads to depletion of host energy reserves.

The brain, which utilizes glucose as its major fuel source, appears to have a reduced supply in the tumour-bearing state which is directly proportional to the utilization of glucose by the tumour (Mulligan and Tisdale, 1991a).

The use of glucose by a tumour at the expense of the

host and the resulting increase in gluconeogenesis is a reasonable explanation for weight loss due to fast-growing and large tumours which may be continually depleting the host's glucose stores but does not explain the increased energy expenditure of the host in response to the presence of small tumours. It also fails to explain why some cachexia-producing tumours do not display high Cori cycle activity (Aisenberg, 1961).

Cancer patients have also shown an abnormal glucose tolerance which has been correlated with a marked resistance to insulin with no apparent change in insulin receptor number or affinity of insulin for its receptor (Schein et al, 1979).

1.2.1.3 Protein metabolism

The total protein content of muscle is decreased in cachectic cancer patients. In tumour-bearing rats, protein synthesis was reduced by 70% in muscle and by 40% in liver (Emery et al, 1984). Incorporation of amino acids into the gastrocnemius muscle protein of tumour-bearing rats has been shown to be decreased compared with non tumour-bearing controls (Clark and Goodlad, 1971). Alterations in amino acid metabolism have also been recorded by Waterhouse et al (1979), these changes being related to the increased hepatic gluconeogenesis from amino acids. Tumours are able to take up amino acids at the expense of the host (Lazo, 1981). However,

the depression of protein synthesis can not be explained merely by a depletion of precursor amino acids. Reduced protein synthesis in tumour cells grown *in vitro* from cancer patients is not completely corrected by the addition of an excess of amino acids (Shamberger, 1984).

A negative nitrogen balance has been observed in many human and animal cancers (Theologides, 1972) indicating loss of lean body tissue. In an experiment with mice bearing the MAC16 colon adenocarcinoma a significant increase in nitrogen excretion in tumour-bearing animals was seen with relatively small weight losses. With larger weight losses conservation of nitrogen was seen with levels of nitrogen similar to those seen in non tumour-bearing animals (Beck and Tisdale, 1989a). The increased nitrogen excretion was not seen in animals bearing the related MAC13 tumour which is of the same histological type as the MAC16 tumour but does not produce cachexia in the host, thus suggesting that the nitrogen loss is not merely due to competition of the tumour for amino acids.

Even in cases where weight loss appears to be due to the presence of a tumour in a particular site rather than the presence of a malignancy, alterations in protein metabolism have been shown. A study conducted by Burke *et al* (1980) on patients with either benign or malignant gastro-intestinal tumours initially showed no significant difference in weight loss and food intake measurements between the two groups of patients. However further investigation revealed significant correlations between protein and energy intakes and the ratio

of total body potassium (indicative of lean body mass) to total body water in patients with benign disease but not in those with cancer.

1.2.1.4 Lipid Metabolism

Increased fat metabolism is an important feature of cachexia. In humans a rapid reduction of total body fat has been reported to accompany a malignancy. An increased energy expenditure with a resultant calorie deficit which could not be corrected with parenteral feeding was seen during that time (Watkin, 1959). A loss of body fat in cachectic cancer patients who did not display anorexia was reported by Warnold et al (1978). The loss of body lipid due to mobilization of free fatty acids from the adipose tissue of cancer patients may occur at an early stage of the disease (Kralovic et al, 1977). A significant increase in the plasma concentration of non-esterified fatty acids (Mueller and Watkin, 1961) and an increase in glycerol turnover (Eden et al, 1985) have both been reported in patients with malignant disease. These observations along with the report of an increased rate of removal of infused lipids from the blood of cancer patients (Waterhouse and Nye, 1961) indicate an increased requirement for energy from this source.

Lundholm et al (1981) reported a loss of carcass fat which was not merely due to decreased food intake since pair-fed rodents did not show similar depletion of lipid stores. A

high fat diet reduced this loss but did not completely reverse it. Buzby et al (1980) discovered that an isocaloric isonitrogenous fat-based total parenteral nutrition regimen produced a maintenance of weight in tumour-bearing rats without the increase in tumour weight seen with a carbohydrate-based regimen. This would seem to indicate a preference for carbohydrate-derived nutrients by the tumour and fat-derived nutrients by the host in the tumour-bearing state. Brain metabolism is maintained by increased utilization of ketone bodies derived from lipid metabolism in the liver (Mulligan and Tisdale, 1991a). This and the increased lipogenesis from glucose in tumour-bearing animals (Mulligan and Tisdale, 1991b) emphasizes the need for lipid metabolites by host tissues.

Alterations in heart and adipose lipoprotein lipase activity have been observed in cancer cachexia with an initial increase in activity preceeding a decrease in both tissues with increasing weight loss (Briddon et al, 1991). The increase in lipoprotein lipase activity in heart tissue appeared to be related to the tumour-bearing state whereas the increase in adipose tissue was specific to the cachectic state. A different cachectic tumour model showed an increase in heart lipoprotein lipase activity along with high levels of activity in tumour tissue, and a decrease in adipose tissue activity. This suggested that plasma triglycerides were being redirected away from storage for use by other tissues including the tumour in the cachectic state (Thompson et al,

1981). The differences in these results make interpretation of the role of lipoprotein lipase in cancer cachexia very difficult.

The suggestion that tumours may adapt to utilize fatty acids as an energy source at the expense of the host was made by Medes et al (1952). These researchers noted that lipogenesis occurs in neoplastic tissues but that this process was probably too slow to supply the fatty acids required by a rapidly growing tumour. They suggested that the tumour obtained lipids which were preformed by the host. In addition to this, Medes et al (1956) suggested that tumours readily accept dietary fatty acids and utilize these as an energy source. A more recent study by Sauer and Dauchy (1987) emphasizes the relationship between host lipid supplies and tumour growth. These researchers noted that an acute fast, which promoted host mobilization of adipose stores, led to stimulation of tumour growth. A related observation was made in animals bearing the MAC16 tumour (Tisdale and Beck, 1991) in which inhibition of fat mobilization was associated with inhibition of tumour growth.

Liebelt et al (1971) suggested that the tumour itself could be producing a lipolytic substance. Since then many catabolic factors have been isolated such as toxohormone L, a lipolytic factor from the cell-free fluid of ascites sarcoma (Masuno et al, 1981) in addition to lipolytic and proteolytic factors isolated from the MAC16 adenocarcinoma (Beck and Tisdale, 1987). More recently a lipoprotein lipase -

inhibiting protein has been isolated from the medium in which a human melanoma cell line had been grown. Mice bearing this tumour develop severe cachexia which may be due to the reduced intake of fatty acids into adipose and muscle tissue (Mori et al, 1989). A 6000Da protein has recently been isolated by Taylor et al (1992). This protein has been shown to produce lipolysis in vitro which correlated with loss of carcass fat in vivo and appears to be related to its ability to induce cellular lipase activity.

It has been suggested that tumour necrosis factor (TNF/cachectin), a substance secreted by macrophages, may be responsible for the weight loss associated with malignant disease (Oliff et al, 1987). TNF was originally isolated based on its ability to inhibit lipoprotein lipase (Beutler et al, 1985). However studies have indicated that TNF is probably responsible for the anorectic effect rather than the cachectic effect seen in tumour-bearing subjects (Mahony et al, 1988).

1.3 The Treatment of Cancer Cachexia

Theologides (1979) stated that although prevention and control of the complications of cancer and its treatment may delay the development or reduce the severity of the cachexia experienced by the cancer patient, only the effective control of the underlying malignancy can reverse this syndrome. This has indeed been shown to be true but the

situation may be viewed from another angle. If a tumour is relying on its host to provide nutrients for its growth either by indirect alterations in host metabolism or by a direct catabolic action due to tumour-derived factors, treatment of the cachexia by selectively starving tumour cells of nutrients while providing nutritional support to the host or directly preventing breakdown of body fat and protein should also lead to an inhibition of tumour growth.

1.3.1 Total Parenteral Nutrition

Early studies investigating the role of total parenteral nutrition (TPN) in the treatment of cancer cachexia suggested that it reduced some of the toxicity of chemotherapy, improved the response rate to treatment and possibly improved the outcome of the disease (Body et al, 1987). An animal study showed TPN to increase the proportion of tumour cells in S-phase (Torosian et al, 1984) which could be useful in maximizing the effects of cycle-specific chemotherapeutic agents. More recent studies have failed to show a benefit, however. Nixon et al (1981) noted an inferior response to TPN in cancer patients compared with non-cancerous controls. Evans et al (1985) showed a reduction of the increased gluconeogenesis seen in cancer patients in addition to a reduction of muscle breakdown but these effects were short-lived. Buzby et al (1980) have noted a stimulation of tumour growth in an animal model of cachexia with certain forms

of TPN and a worsening of condition in cancer patients has also been noted by Chlebowski (1986). The benefits of long term treatment of cancer cachexia with total parenteral nutrition are therefore doubtful but in the short term it may produce a temporary improvement in the patient's condition and therefore improve the tolerance and response to treatment (Buzby et al, 1980).

1.3.2 High Fat/Ketogenic Diet

The state of ketonaemia which would be expected due to an excess of fatty acids with respect to glucose is very rare in cancer cachexia. This lack of ketosis in cancer does not appear to be due to an impairment in the liver's ability to synthesize ketone bodies since an exogenous source of fatty acids leads to an increase in blood ketone levels (Conyers et al, 1979). Many tumours are deficient in enzymes necessary for the utilization of ketone bodies as a metabolic fuel which may provide the opportunity to replenish energy sources in the host at the expense of the tumour (Tisdale and Brennan, 1986).

In a study by Tisdale et al (1987), mice bearing the MAC16 adenocarcinoma were fed diets with varying fat content (proportions of medium chain triglyceride (MCT) up to 80%). An increase in weight was seen in proportion to the fat content of the diet along with a reduction in blood levels of free fatty acids. A reduction in tumour size also occurred. These effects seemed to be directly attributed to the ketone bodies

generated by this diet since a non-ketogenic diet consisting of long chain triglycerides showed none of these effects (Tisdale and Brennan, 1988). In addition to this, 3-hydroxybutyrate has been shown to inhibit the lipolytic and proteolytic factors produced by the MAC16 tumour *in vitro* (Beck and Tisdale, 1987).

An isocaloric isonitrogenous diet consisting of 80% MCT was shown to restore the nitrogen balance and urea excretion to that of non tumour-bearing controls in addition to raising the levels of depleted amino acids (Beck and Tisdale, 1989a). However, an improvement in nitrogen balance was not seen in cachectic cancer patients fed a ketogenic diet when compared with those fed a normal diet despite an increase in body weight due to the ketogenic diet (Fearon *et al*, 1988).

1.3.3 Insulin

Insulin has been suggested as an anti-cachectic agent due to its anabolic effects. It has been shown to inhibit lipolysis, increase amino acid uptake into muscle protein, inhibit hepatic gluconeogenesis thus restoring the homeostasis of circulating amino acids and decrease proteolysis (cited by Schein *et al*, 1979).

In a study by Moley *et al* (1985), rats with a transplantable sarcoma were given insulin and experienced a significant increase in weight and food intake with no effect

on tumour growth. However when insulin therapy was compared with a ketogenic diet in mice bearing the MAC16 colon adenocarcinoma (Beck and Tisdale, 1989b), although significant increases in weight occurred in both cases, which were unrelated to food intake, tumour growth was increased by 50% with the insulin therapy. This stimulation of tumour growth could be inhibited by 3-hydroxybutyrate with no alteration in the extent of the weight loss. The negative nitrogen balance in animals bearing the MAC16 tumour was reversed with a ketogenic diet but not with insulin administration. The stimulation of tumour growth in addition to insulin resistance in tumour-bearing hosts therefore limits the use of this agent in the clinical management of cachexia.

1.3.4 Specific Inhibitors of Lipolysis as a Treatment for Cancer Cachexia

Efforts to reverse the devastating syndrome of cachexia in cancer patients has in the past involved attempts to replenish depleted host metabolic pools and reverse the metabolic chaos resulting from this condition. However the fact that so many processes are involved and attempts to differentially feed the host at the expense of the tumour carry some risk explains the lack of complete success in this area of research. By aiming at a specific tissue which is believed to be supplying the tumour with nutrients, it may be possible to

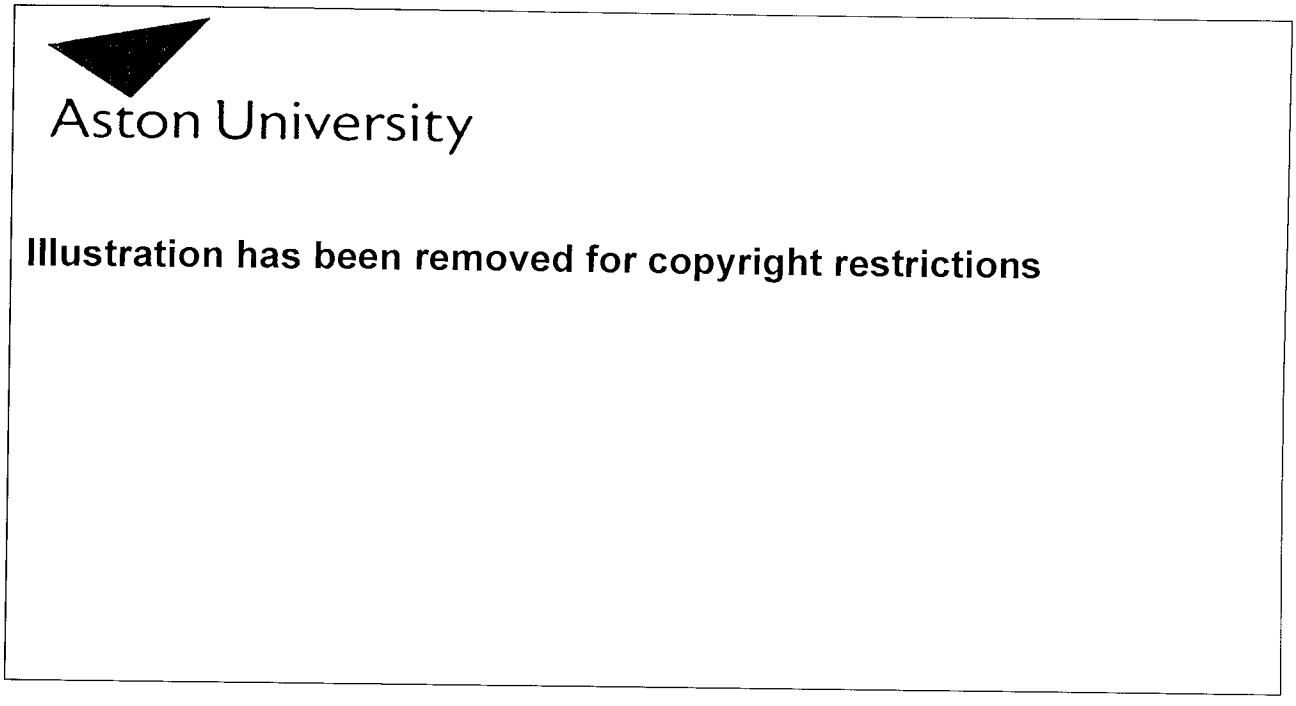
starve the tumour whilst conserving host tissues. If the tumour is secreting catabolic substances which act directly on host adipose or muscle tissue, this pursuit appears to be further justified.

Protein turnover in muscle tissue is a complex process and the exact mechanism by which this is controlled has not been elucidated. The control of lipid metabolism is much better understood and therefore the adipocyte is a useful model for assessing direct molecular interactions of substances which affect the lipolytic aspect of cancer cachexia.

1.4 Hormonal Control of Lipolysis

The mobilization of lipid stores in the adipocyte (fig. 1.3) is initiated by the cleavage of ester bonds in triglycerides by hormone-sensitive lipase which is activated by the cascade mechanism shown in fig. 1.4. Evidence to suggest the link between hormonal stimulation of lipolysis and the phosphorylation (and subsequent activation) of hormone-sensitive lipase was provided by Strålfors et al (1984). This group of researchers discovered two phosphorylation sites on the hormone-sensitive lipase, one (a "basal site") which was phosphorylated in the presence and absence of hormonal stimulation, and another (a "regulatory site") which was phosphorylated by cAMP-dependent protein kinase. Anti-lipolytic agents inhibited phosphorylation of the regulatory

Figure 1.3



Aston University
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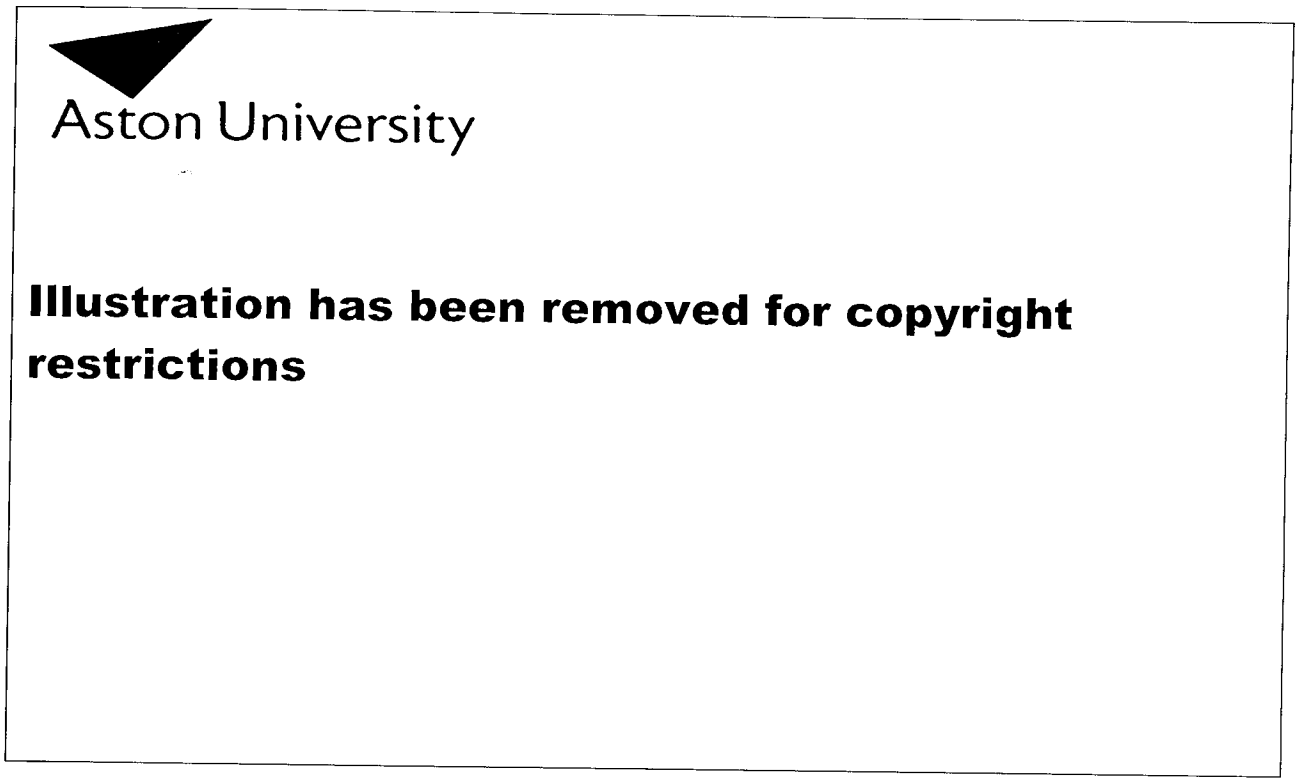
Fatty Acid Cycling in Adipose Tissue

Stryer, 1981

Figure 1.4



Control of Hormone-Sensitive Triglyceride Lipase

Gurr and James,1975

site. This was emphasized by Nilsson et al (1980) who discovered that catecholamine-mediated stimulation and insulin-mediated inhibition of phosphorylation appeared to correlate with stimulation and inhibition respectively of lipolysis in intact adipocytes.

The increase in cyclic adenosine 3',5'-monophosphate (cAMP) necessary to activate cAMP-dependent protein kinase and elicit the phosphorylation of hormone-sensitive lipase in response to hormone receptor stimulation is mediated by a GTP-binding protein which will be discussed in greater detail in subsequent sections.

1.5 Elucidation of the Components of Signal Transduction in Cell Membranes

Early studies by Limbird and Lefkowitz (1977) and Haga et al (1977) into the structure and function of the hormone receptor - adenylate cyclase pathway determined the existence of two distinct proteins; one providing a binding site for β-adrenergic agonists and one possessing catalytic activity within the cell. Ross et al (1978) discovered that this catalytic component could itself be resolved into two components; a thermolabile protein which was suggested to be the catalytic unit and a thermostable protein, also believed to consist of two components which were differentiated according to their functional properties although they were not physically separated. Reconstitution of membranes from a

variant of the S49 lymphoma cell line, the adenylate cyclase-deficient (cyc^-) variant, which lacks the regulatory component of the adenylate cyclase pathway and hence is devoid of hormone-stimulated adenylate cyclase activity, with the solubilized regulatory component of fully competent S49-wild type lymphoma cells was found to restore the hormone-stimulated adenylate cyclase activity seen in S49-wild type cells to this variant (Sternweis and Gilman, 1978). The catalytic component of the hormone-stimulated adenylate cyclase system is now known to be adenylate cyclase itself and the regulatory component is a guanine nucleotide - binding (G) protein.

1.5.1 The Discovery and Isolation of Guanine Nucleotide - Binding Proteins

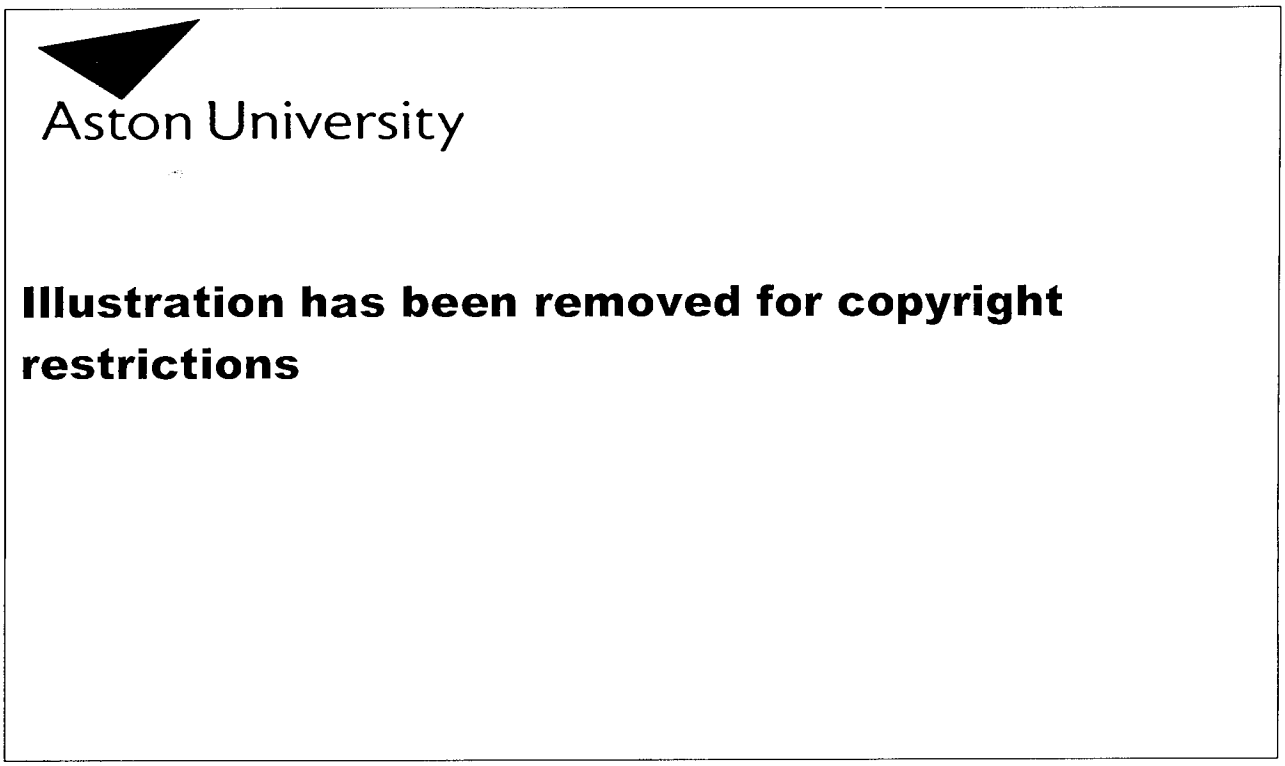
Rodbell et al (1971) were the first to discover the specific requirement for guanine nucleotides in hormonal function. Cassel and Selinger (1976) suggested the relationship between catecholamine-induced stimulation of adenylate cyclase activity and the hydrolysis of guanosine 5'-triphosphate. This catecholamine-stimulated GTP-ase activity was blocked by propranolol but not by inactivation of the adenylate cyclase molecule or addition of cAMP, thus indicating that the GTP-ase activity was independent of the catalytic production of cAMP by adenylate cyclase. Ross et al (1977) discovered that β-agonists were unable to stimulate

adenylate cyclase of S49 lymphoma plasma membranes alone and that although the addition of certain purine nucleotides decreased the binding of β-agonists to their receptor, when added in a concentration which was insufficient to significantly alter the binding affinity of the β-agonist, a stimulation of adenylate cyclase with a K_{act} comparable to the K_d of the agonist-receptor interaction was attained. This strengthened the suggestion of the requirement for purine nucleotides in receptor-mediated adenylate cyclase activity.

Much is now known about the family of guanine nucleotide - binding proteins involved in signal transduction. It is now known that distinct guanine nucleotide - binding proteins exist which exert inhibitory as well as stimulatory effects on effector molecules. A GTP-dependent inhibitory effect on adenylate cyclase activity was found to exist in S49 cyc^- lymphoma cells, which do not contain G_s, and therefore could not be due to inhibition of this regulatory component (Hildebrandt et al, 1983; Jakobs et al, 1983). All G-proteins are heterotrimeric in structure (fig. 1.5) and comprise an α-subunit which binds guanine nucleotides in addition to β and γ-subunits which always appear to be closely associated. The function of the β and γ-subunits is not yet fully understood but it appears that they may be directly involved in the mechanism of action of inhibitory G-proteins (Katada et al, 1987).

The α-subunit possesses intrinsic GTP-ase activity

Figure 1.5



G-Protein Structure

Litwack, 1992

and is thought to determine the stimulatory or inhibitory nature of the G-protein in a particular pathway. The α-subunits so far discovered range in molecular weight from 39 - 52 kDa. The primary amino acid sequences and overall tertiary structures appear to be very highly conserved and attempts to differentiate between the different sub-types rely on immunochemical techniques using specific antisera and the ability of the α-subunit to undergo ADP-ribosylation catalysed by bacterial toxins (Milligan, 1988). Many of the α-subunits so far discovered have been shown to affect a particular effector molecule. However there are a few which have been isolated whose function is as yet unknown (eg. Katada et al, 1987; Milligan, 1987), just as there are GTP-dependent pathways thought to involve G-proteins which have yet to be identified (Taylor, 1990).

The β-subunits so far isolated vary in molecular weight from 35 - 36 kDa. Contrary to original assumptions that all β-subunits within a particular tissue possess the same structure, more recent evidence suggests the existence of at least two physically but not functionally distinct forms (Sternweis and Robishaw, 1984; Taylor, 1990).

The γ-subunit has a molecular weight of around 10 kDa and recent studies indicate the existence of three distinct forms (Taylor, 1990).

1.5.2 Mechanism of Action of Guanine Nucleotide - Binding Proteins

The process by which a G-protein mediates in the activation of a physiological process by a hormone or drug comprises several stages which are summarized below and in fig. 1.6 (Litwack, 1992).

The first step involves the binding of the hormone to its receptor. This induces a conformational change in the receptor which subsequently binds to the G-protein. GDP bound to the α-subunit is exchanged for GTP which yields the active form of the G-protein. The subunits dissociate and the α-subunit binds to the inactive effector molecule, activates it and causes an alteration in intracellular physiological events. The process is terminated when GTP is hydrolysed to GDP, causing the α-subunit to dissociate from the effector molecule and bind to the βγ complex. The G-protein is once again in its inactive form and can be reactivated by another receptor-hormone complex.

Less is known about the role of G-proteins in inhibitory mechanisms. It is thought that the binding of the dissociated α-subunit to the effector molecule produces a conformational change in that molecule which inhibits its action. Alternatively the released βγ complex could be responsible for the inhibitory effect. All βγ complexes are functionally very similar, if not identical, and can interact with a wide range of α-subunits. This prompted the theory that

Figure 1.6

Hormonal Control of Adenylate Cyclase

Litwack, 1992

$\beta\gamma$ released from G_i can combine with and inactivate $G_s\alpha$ by shifting the equilibrium towards the inactive GDP-bound form of G_s (Hardie, 1991). This does not, however, explain the ability of hormones to inhibit adenylate cyclase in the cyc^- mutant of S49 lymphoma cells which are devoid of $G_s\alpha$. Another explanation is that $\beta\gamma$ exerts a direct inhibitory action on the effector molecule (Katada et al, 1987)

1.5.3 Characterization of Guanine Nucleotide - Binding Proteins Using Bacterial Toxins

Bacterial toxins have been used for many years as tools in the characterization of guanine nucleotide - binding (G) proteins, in particular those regulating the adenylate cyclase system (Milligan, 1988).

1.5.3.1 Cholera Toxin

Cholera toxin is derived from the bacterium Vibrio cholerae and consists of two subunits. The A subunit is the active part of the toxin and the B subunit is responsible for binding of the toxin to the cell surface and promoting the entry of the active subunit into the cell.

Cholera toxin catalyses the transfer of an ADP-ribose unit from NAD^+ to a conserved arginine residue in the α-subunit of certain G-proteins. The α-subunit catalyses the hydrolysis of GTP to GDP and on treatment with cholera toxin the GTP-bound

form of this polypeptide is stabilized leading to a permanently activated form of the G-protein.

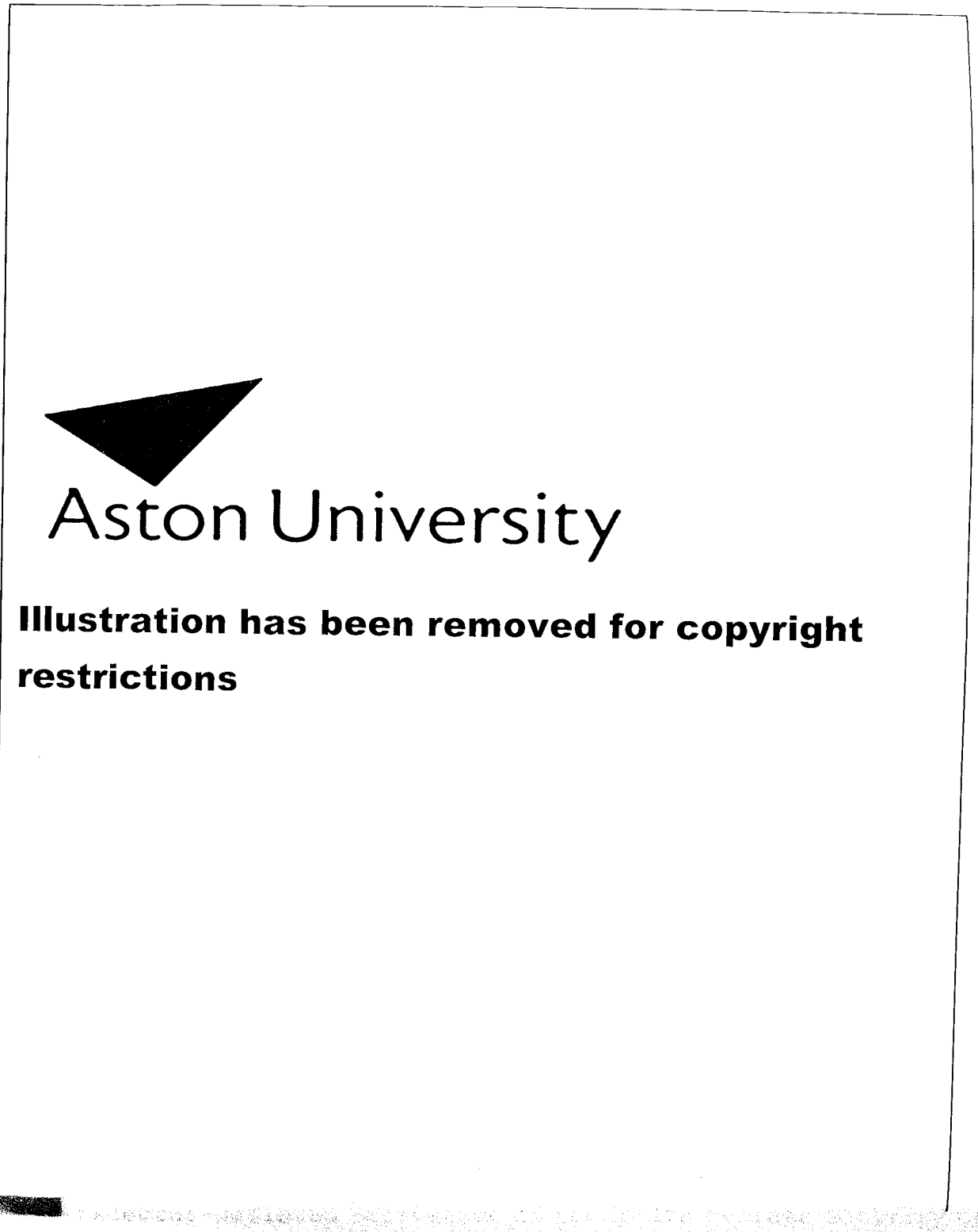
The substrates for cholera toxin are, in the main, those which exert a stimulatory effect. They include the two forms of the α-subunit of G_s, the stimulatory G-protein in the adenylate cyclase pathway, which have molecular weights of around 45 and 52 kDa. Each of these polypeptides is also believed to exist in two forms and all four forms are believed to be derived from a single gene (Bray et al, 1986; Robishaw et al, 1986). The light transducing proteins of the retina, T1 and T2 (present in rods and cones respectively) are also substrates for cholera toxin.

1.5.3.2 Pertussis Toxin

Pertussis toxin, from the bacterium Bordetella pertussis, comprises five distinct subunits, one of which is the active component of the toxin. The remaining subunits are responsible for binding of the toxin and transportation of the active subunit into the cell.

Pertussis toxin catalyses ADP-ribosylation of the α-subunit of some G-proteins (fig. 1.7). In this case substrates appear to be α-subunits of G-proteins which generally exert control of an inhibitory nature (Murayama and Ui, 1983). An exception is the case of the transducins, the G-proteins involved in the transduction of light signals through the retina. The Tlα- and T2α-subunits of the G-proteins present in

Figure 1.7



Pertussis Toxin - Catalysed ADP-Ribosylation of Gi

Hardie, 1991

rods and cones respectively are responsive to pertussis toxin and cholera toxin, yet both exert a stimulatory effect on cyclic GMP phosphodiesterase which is the effector molecule in this system (Taylor, 1990).

The consequence of the interaction of pertussis toxin with the α-subunit of susceptible proteins is the removal of the inhibition due to these G-proteins. Whereas the site of action for cholera toxin is an arginine residue, pertussis toxin binds to a cysteine residue located four amino acids from the C-terminus of the α-subunit (Milligan, 1988). This binding site is present in $G_{i-1,2 \text{ and } 3}\alpha$, $G_o\alpha$, T1α and T2α subunits. G_i is the inhibitory G-protein controlling adenylate cyclase activity. Three subtypes of the α-subunit of this protein have been isolated and many tissues contain more than one subtype (Hinsch et al, 1988; Jones and Reed, 1987; Bushfield et al, 1990). $G_o\alpha$ is the most abundant G-protein subunit in brain tissue. It exists in at least two distinct forms with molecular weights of around 39 and 41 kDa (Sternweis and Robishaw, 1984).

1.5.4 G-Proteins Regulating Adenylate Cyclase Activity in Adipocytes

Murayama and Ui (1983) observed a GTP-dependent receptor-mediated activation of adenylate cyclase activity in rat adipocyte plasma membranes. An inhibitory effect was also noted which was abolished by treatment with pertussis toxin

and was associated with the ADP-ribosylation of a 41 kDa protein within the membrane.

Hinsch et al (1988) reported that the rat adipocyte possessed two cholera toxin substrates with molecular weights of 42 and 43 kDa (corresponding to the two forms of G_s) and two substrates for pertussis toxin with molecular weights of 40 and 41 kDa (corresponding to two forms of G_i). Immunochemical techniques eliminated the possibility that one of these pertussis toxin substrates was G_o.

More recently, Mitchell et al (1989) have demonstrated the presence of three forms of G_i in rat adipocytes, namely $G_{i-1}\alpha$, $G_{i-2}\alpha$ and $G_{i-3}\alpha$ which have apparent molecular weights of 41, 41 and 40 kDa respectively (Graziano and Gilman, 1987).

Ohisalo and Milligan (1989) identified a 40 kDa protein in human adipocytes which was a substrate for pertussis toxin catalysed ADP-ribosylation. Two cholera toxin - sensitive proteins of molecular weights 42 and 45 kDa were also detected with the former being more prominent.

1.6 Aims of The Study

Lipolytic and proteolytic factors produced by the murine MAC16 colon adenocarcinoma have been shown to produce a decrease in host fat and lean body tissue which is directly proportional to the weight of the tumour (Beck and Tisdale, 1987). Chromatographic analysis of tumour and serum samples

from animals bearing this tumour showed peaks of lipolytic activity corresponding to molecular weights of approximately 3.0, 1.5 and 0.7 kDa. The same peaks of activity were observed with samples from some human tumours. The object of the first part of this study was to further characterize the lipolytic factor produced by the MAC16 tumour *in vivo* and *in vitro*.

Following the characterization of the MAC16 lipolytic factor, the second part of this study was an attempt to elucidate the mechanism of action of two drugs shown to exhibit anti-cachectic activity *in vivo*, specifically looking at their effect on lipolysis.

The main subject of this investigation was the ω-3 polyunsaturated fatty acid, eicosapentaenoic acid (EPA), which is a major constituent of fish oil. This fatty acid has demonstrated anti-tumour and anti-cachectic activity *in vivo* in addition to anti-lipolytic activity *in vitro* (Tisdale and Beck, 1991).

Recent research has suggested the link between prostaglandins and cancer (Karmali *et al*, 1984; Tashjian *et al*, 1984). The ω-3 fatty acid constituents of fish oil, eicosapentaenoic acid and docosahexaenoic acid are both inhibitors of 2-series prostaglandin synthesis (Culp *et al*, 1979; Corey *et al*, 1983). Thus the effect of eicosapentaenoic acid and docosahexaenoic acid, which does not exhibit an anti-tumour or anti-cachectic effect in tumour-bearing animals (Tisdale and Beck, 1991), on prostaglandin production in a murine cachexia model was investigated.

The observation that EPA caused a decrease in hormone and lipolytic factor - stimulated cAMP increases in intact adipocytes (Tisdale and Beck, 1991) suggested the involvement of G_s and G_i, the stimulatory and inhibitory G-proteins involved in the adenylate cyclase system, in the mechanism of action of the lipolytic factor and EPA respectively. Thus the effect of EPA on G_i in adipocyte plasma membranes was investigated. The effect of EPA on calcium mobilization induced by f-Met-Leu-Phe in the human leukaemic HL60 cell line was also investigated in order to seek a G-protein mediated process common to both signal transduction systems and/or a potential for intracellular cross-talk between the adenylate cyclase and phosphoinositide systems.

To conclude the investigation of EPA, a detailed examination of its effect on adenylate cyclase activity was conducted. A comparison was made with docosahexaenoic acid and the ω-6 fatty acid, arachidonic acid, and the relationship between the action of these fatty acids on adipocyte adenylate cyclase and lipolysis was determined.

The final part of this study was a preliminary investigation into the anti-cachectic action of N-(3-phenoxycinnamyl)acetohydroxamic acid (BW A4C). This compound is an inhibitor of 5-lipoxygenase and interest in a possible anti-cachectic effect of the drug was originally aroused by suggestions that lipoxygenase products could be involved in cancer (Buckman et al, 1991). An inhibitory effect on tumour growth and associated weight loss has been demonstrated in

animals bearing the MAC16 tumour (Beck, S.A. and Hudson, E.A., unpublished results) which led to this investigation of a possible direct inhibition of adipocyte lipolysis.

The overall aim of this investigation was to further characterize the tumour-derived lipolytic factor originally reported by Beck and Tisdale (1987) and to investigate possible treatments for cancer cachexia which specifically inhibit the action of this factor at the level of the adipocyte.

SECTION 2 : MATERIALS

2.1 ANIMALS

Pure strain NMRI and BKW mice (age 12-15 weeks) were purchased from Bantin and Kingman, Hull, UK. Rat and mouse breeding diet was obtained from Pilsbury's Ltd, Birmingham, UK.

2.2 CHEMICALS

The following compounds were obtained from:

AMERSHAM INTERNATIONAL, Amersham, Bucks, UK

[8-^{3}H]Adenosine 3',5'-cyclic phosphate, ammonium salt
(Specific activity 21.2 Ci/mmol)

[2-^{3}H]Adenosine 5'-monophosphate, ammonium salt
(Specific activity 23 Ci/mmol)

[^{14}C]Methylated protein molecular weight markers

BDH LIMITED, Poole, England

Acetone

N,N'-Bis-methylene acrylamide

Dipotassium hydrogen orthophosphate

Ethylenediaminetetraacetic acid, disodium salt

Methanol

Potassium dihydrogen orthophosphate

Sodium pyruvate

Sodium succinate

BIO-RAD LABORATORIES, Richmond, CA

Ammonium persulphate

Bradford Reagent

DUPONT, NEN Division, Dreieich, Germany

Aquasol

[5,6,8,11,12,14,15-^{3}H(N)]Prostaglandin E_2

(Specific activity 200 Ci/mmol)

FISONS SCIENTIFIC APPARATUS, Loughborough, UK

Acrylamide

Barium hydroxide

Chloroform

Diethylether

Ethanol

Ethyl acetate

Hydrochloric acid

Folin and Ciocalteu's phenol reagent

Glacial acetic acid

Magnesium chloride

Magnesium sulphate

Optiphase Hisafe 3

Perchloric acid

Potassium cyanide

Sodium carbonate

Sodium chloride

Sodium dihydrogen orthophosphate

Sodium dodecyl sulphate

Sodium hydroxide

Sucrose

FLOW LABORATORIES LTD, Rickmansworth, Herts, UK

Human Plasma, AB^+

Trypan blue

GIBCO LTD, Paisley, Scotland

RPMI 1640 medium

ICN FLOW, High Wycombe, Bucks, UK

[α-^{32}P]Adenosine 5'-triphosphate
(Specific activity 3000 Ci/mmol)

[Adenylate-^{32}P]Nicotinamide adenine dinucleotide,
dipotassium salt (Specific activity 250 Ci/mmol)

IMPERIAL LABORATORIES LTD, Andover, Hants, UK

Foetal calf serum

OXOID, Basingstoke, Hants, UK

Complement fixation buffer tablets

Phosphate buffered saline tablets

SCOTIA PHARMACEUTICALS LTD., Guildford, Surrey, UK

DHA 99 (99% DHA, 1% other fatty acids)

EPA 80 (80% EPA,10% DHA,10% other fatty acids)

GLA 99 (99% GLA, 1% other fatty acids)

SIGMA CHEMICAL COMPANY, Poole, Dorset, UK

Activated charcoal

Adenosine 3',5'-cyclic phosphate

Adenosine 5'-monophosphate, sodium salt

Adenosine-5'-triphosphate, trisodium salt

Alumina WN-3 neutral resin

Arachidonic acid

Arginine

Bovine serum albumin

Bovine γ-globulin

Bromophenol blue

Calcium chloride

Collagenase

Coomassie brilliant blue

Copper sulphate

Creatine phosphate

Creatine phosphokinase

Cytochrome c

Dextran

Dextran blue

N^6,2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate

2,6-Dichlorophenolindophenol, sodium salt

Dimethylsulphoxide

Dithiothreitol

Disodium hydrogen orthophosphate

Docosahexaenoic acid

Dowex 50WX8-400 resin

Eicosapentaenoic acid

Ethidium bromide

Ethylene glycol - bis (B-aminoethyl ether) N,N,N',N'-tetraacetic acid

Formylmethionylleucylphenylalanine

Forskolin

Glucose

Glutamine

Glycerokinase

Glycine

Guanosine 5'-triphosphate

N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid

Imidazole

Indomethacin

3-Isobutyl-1-methylxanthine

Isoprenaline

Kodak GBX Developer and Replenisher

Kodak GBX Fixer and Replenisher

Lactate dehydrogenase

Lysine

Nicotinamide adenine dinucleotide

Nicotinamide adenine dinucleotide, reduced form

Nitrobluetetrazolium

Percoll

Pertussis toxin

Phosphoenolpyruvate

Potassium chloride

Potassium hydroxide

Prostaglandin E_2

Prostaglandin E_2 antiserum

Pyruvate kinase

Quin 2-acetoxymethyl ester

Rabbit serum

Sephadex G50

Sodium azide

Sodium bicarbonate

Sodium/potassium tartrate

12-O-Tetradecanoylphorbol-13-acetate

N,N,N',N'- Tetramethylethylenediamine

Thymidine

Toluidine blue O

Triethanolamine

Tris(hydroxymethyl)methylamine

Triton X-100

Urea

Zinc sulphate

THE WELLCOME FOUNDATION Ltd, Crewe, UK

BW A4C

WHATMAN, Maidstone, Kent, UK

DEAE cellulose

2.3 BUFFERS

2.3.1 Krebs Ringer Bicarbonate Solution

Sodium chloride	118mM
Potassium chloride	5mM
Calcium chloride	2mM
Potassium dihydrogen orthophosphate	1mM
Magnesium sulphate	1mM
Sodium bicarbonate	25mM

2.3.2 Krebs Buffer, pH 7.6

Bovine serum albumin	3.0g
D-glucose	9.91mg
Krebs Ringer Bicarbonate Solution	to 100ml

2.3.3 Glycerol Buffer

Triethanolamine	100mM
Magnesium sulphate	2mM
Phosphoenolpyruvate	0.4mM
NADH	0.25mM
ATP	1.2mM
Pyruvate kinase	1 unit/ml
Lactate dehydrogenase	7 units/ml

The pH was adjusted to 7.4 with concentrated hydrochloric acid and the solution made up to volume with deionized water.

2.3.4 Neutrophil Buffer, pH 7.2

NaCl	140mM
KCl	5mM
$NaHCO_3$	2.8mM
$CaCl_2$	1.5mM
$MgCl_2$	0.06mM
$MgSO_4$	0.6mM
Glucose	5.6mM
N-2-Hydroxyethylpiperazine-N-2 -ethanesulphonic acid	15mM

The pH was adjusted with 1M NaOH and the solution made up to volume using deionized water.

2.3.5 Complement Fixation Buffer

Oxoid CFB tablet	1 tablet
Distilled water	to 1000ml

This gives a solution containing 0.575g barbitone, 0.185g soluble barbitone, 8.5g sodium chloride, 0.168g magnesium chloride, 0.028g calcium chloride/litre, pH 7.4.

2.3.6 PG Buffer

NaCl	0.877g
Sodium azide	0.1g
Bovine serum albumin	0.1g
10mM Sodium Phosphate Buffer, pH 7.4	to 100ml

2.3.7 Dextran - Coated Charcoal Suspension

Activated charcoal	0.5g
Dextran	0.05g
PG Buffer	to 50ml

2.3.8 Sucrose Buffer

Sucrose	0.25M
EGTA	2mM
Tris-HCl, pH 7.4	10mM

2.3.9 Concentrated Sucrose Solution

Sucrose	2M
EGTA	8mM
Tris-HCl, pH 7.4	80mM

2.3.10 NaCl Buffer

NaCl	0.15M
EGTA	1mM
Tris-HCl, pH 7.4	10mM

2.3.11 30% Acrylamide/Bis Stock Solution

Acrylamide	30g
N,N'-Bis-methylene acrylamide	0.8g
Distilled water	to 100ml

The solution was filtered and stored in the dark.

2.3.12 Running Gel Buffer

Tris	19.2g
Sodium dodecyl sulphate	0.4g
Distilled water	to 100ml

The pH was adjusted to 8.7 with concentrated hydrochloric acid.

2.3.13 Running Gel, 12%

30% Acrylamide/Bis Stock Solution	6ml
Running Gel Buffer	3.75ml
Distilled water	5.1ml
Ammonium persulphate, 10%w/v	150µl
TEMED	10µl

2.3.14 Stacking Gel Buffer

Tris	6.06g
Sodium dodecyl sulphate	0.4g
Distilled water	to 100ml

The pH was adjusted to 6.8 with concentrated hydrochloric acid.

2.3.15 Stacking Gel, 5%

30% Acrylamide/Bis Stock Solution	1.65ml
Stacking Gel Buffer	2.5ml
Distilled water	5.75ml
Ammonium persulphate, 10%w/v	100µl
TEMED	7µl

2.3.16 Electrolyte Buffer

Tris	3.03g
Glycine	14.41g
Sodium dodecyl sulphate	1g
Distilled water	to 1000ml

The pH was adjusted to 8.3 with concentrated hydrochloric acid.

2.3.17 Laemmli Sample Buffer

Sodium dodecyl sulphate 20%w/v solution	1ml
1M Tris-HCl, pH7.0	0.5ml
60%w/v Sucrose solution	0.5ml
Distilled water	8ml

1mg bromophenol blue and 2.5mg/ml dithiothreitol was added to the solution immediately prior to use.

2.3.18 Coomassie Blue Gel Stain

Coomassie blue R250 stain	2.5g
Methanol	500ml
Glacial acetic acid	100ml
Distilled Water	400ml

2.3.19 Gel Destain

Methanol	150ml
Glacial acetic acid	100ml
Distilled water	750ml

2.3.20 Phosphate Buffers

2.3.20.1 10mM Potassium Phosphate Buffer, pH 8.0

Solution A:	Dipotassium hydrogen orthophosphate	2.28g
	Distilled water	to 1000ml
Solution B:	Potassium dihydrogen orthophosphate	1.36g
	Distilled water	to 1000ml

Solution B was added to Solution A until pH 8.0 was obtained.

2.3.20.2 10mM Potassium Phosphate Buffer, pH7.4

Solution A:	Dipotassium hydrogen orthophosphate	0.228g
	Distilled water	to 100ml
Solution B:	Potassium dihydrogen orthophosphate	0.136g
	Distilled water	to 100ml

Solution B was added to Solution A until pH 7.4 was obtained.

2.3.20.3 500mM Potassium Phosphate Buffer, pH 7.4

Solution A:	Dipotassium hydrogen orthophosphate	57.1g
	Distilled water	to 500ml
Solution B:	Potassium dihydrogen orthophosphate	34.0g
	Distilled water	to 500ml

Solution B was added to Solution A until pH 7.4 was obtained.

2.3.20.4 25mM Sodium Phosphate Buffer, pH 6.8

Solution A:	Disodium hydrogen orthophosphate	0.445g
	Distilled water	to 100ml
Solution B:	Sodium dihydrogen orthophosphate	0.390g
	Distilled water	to 100ml

Solution B was added to Solution A until pH 6.8 was obtained.

2.3.20.5 50mM Potassium Phosphate Buffer, pH 7.5

Solution A:	Dipotassium hydrogen orthphosphate	1.14g
	Distilled water	to 100ml
Solution B:	Potassium dihydrogen orthophosphate	0.68g
	Distilled water	to 100ml

Solution B was added to Solution A until pH 7.5 was obtained.

2.3.20.6 10mM Sodium Phosphate Buffer, pH 7.4

Solution A:	Disodium hydrogen orthophosphate	0.178g
	Distilled water	to 100ml
Solution B:	Sodium dihydrogen orthophosphate	0.156g
	Distilled water	to 100ml

Solution B was added to Solution A until pH 7.4 was obtained.

2.3.20.7 200mM Sodium Phosphate Buffer, pH 7.4

Solution A:	Disodium hydrogen orthophosphate	3.56g
	Distilled water	to 100ml
Solution B:	Sodium dihydrogen orthophosphate	3.12g
	Distilled water	to 100ml

Solution B was added to Solution A until pH 7.4 was obtained.

SECTION 3 : METHODS

3.1 In Vivo Characterization of the MAC16 Colon Adenocarcinoma

3.1.1 Determination of the Relationship Between Serum Lipolytic Activity and Time After Transplantation

3.1.1.1 Transplantation of MAC16 Solid Tumours and Collection of Blood Samples

The MAC16 adenocarcinoma was originally induced with 1,2-dimethylhydrazine by Dr J. Double, Bradford University (Bibby et al, 1987). The tumour was transplanted by excision from a donor animal followed by subcutaneous implantation of small fragments (1 × 2mm in size) in sterile isotonic saline into the flank of the right limb of NMRI mice (Mr. M. Wynter, Aston University). The MAC16 tumour had a doubling time of 3 - 4 days (as determined by Mr. M. Wynter, Aston University) and produced cachexia in the host animal. Mice were weighed daily during the experiment and sacrificed before weight loss reached 30% of the original body weight or if tumours ulcerated. Weight loss began approximately 14 days after transplantation. At this point, after daily weighing of the mice, blood samples were collected from the tail vein. These samples were kept on ice for 1 hour and spun down in a Heraeus Sepatech Biofuge 13 to obtain serum, which was removed and frozen at -20°C prior to assay (5µl) for lipolytic activity (see Section 3.1.1.2). Carcasses were stored at -20°C until

required for body composition analysis.

3.1.1.2 Lipolysis Assay

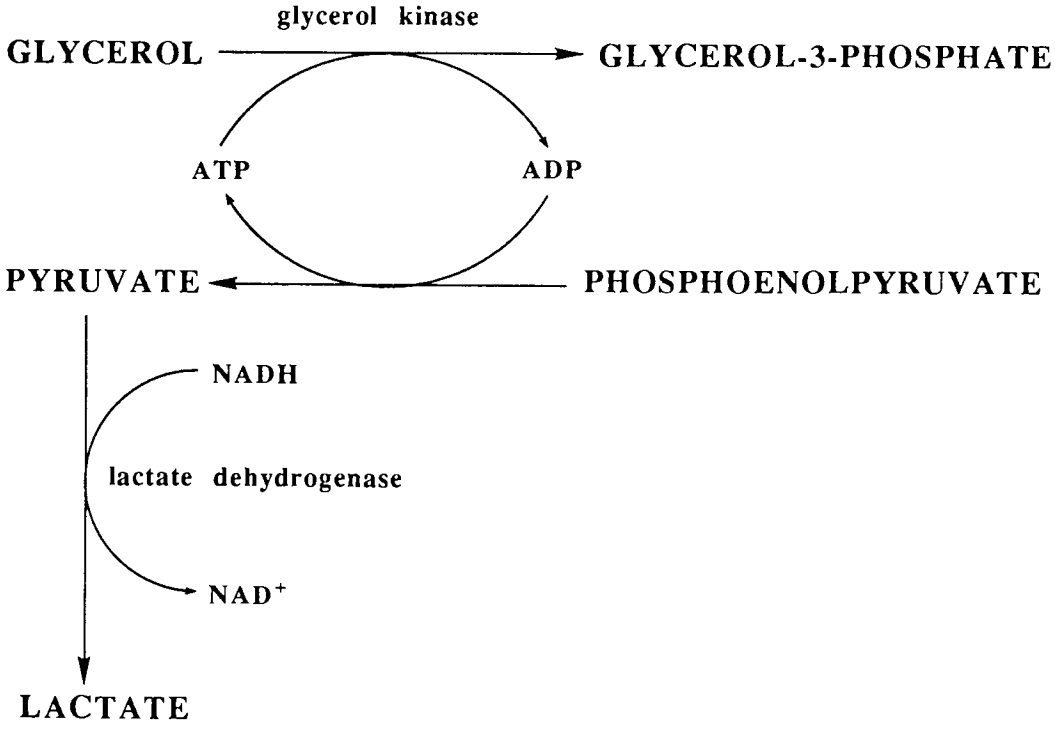
Epididymal fat pads were removed from BKW mice and each pair was placed in a 1ml aliquot of Krebs Buffer pH 7.6 (Section 2.3.2), containing collagenase 2mg/ml. The fat pads were minced, gassed for approximately 15 sec with 95% O_2/5% CO_2 and incubated at 37°C for 2 hr. The isolated fat cells were pooled in a clean universal flask and washed twice in Krebs Ringer Bicarbonate Solution (Section 2.3.1) in order to remove the collagenase. After the final wash the fat cells were diluted with a small amount of Krebs Buffer and counted using a Neubauer haemocytometer. The cell suspension was further diluted to give a final concentration of approximately 1×10^5 adipocytes/ml. 1ml fat cell suspension was added to each of the sample tubes which were then gassed with 95% O_2/5% CO_2 and incubated for 2 hr at 37°C. Sample controls consisted of the sample plus 1ml Krebs Buffer. Fat cell controls consisted of 1ml fat cell suspension plus a volume of Krebs Buffer equivalent to that of the samples. After the 2 hr incubation 0.5ml of each incubation mixture was deproteinized with 0.5ml 10% perchloric acid. The supernatant obtained after centrifugation was neutralized with potassium hydroxide (40%w/v), centrifuged to remove the precipitated potassium perchlorate and the resulting samples were assayed (5µl) for glycerol content, either immediately or after freezing at

-20°C for up to 72 hr.

3.1.1.3 Glycerol Assay

This spectrophotometric assay gives a measurement of the amount of glycerol present in a sample by means of the following reaction.

Figure 3.1



The conversion of NADH to NAD^+ causes a decrease in absorbance which is proportional to the number of glycerol molecules in the sample.

A sample (200µl, obtained from lipolysis assay) was placed into a cuvette with Glycerol Buffer (0.83ml) (Section 2.3.3). The reaction was initiated by the addition of glycerol kinase (1 unit) and the optical density was read at 340nm for 25 min at a temperature of 25°C on a Beckman DU-70 spectrophotometer. The quantity of glycerol was calculated assuming the molar quantity of NAD^+ produced from NADH to be equivalent to that of glycerol present in the sample.

3.1.2 Body Composition Analysis

The gastrocnemius and thigh muscles were removed from the left hind leg of the carcass and the carcass wet weight was recorded. The carcass and muscles were then placed in an oven at 80°C and removed when a constant weight had been achieved. The dry weight of the carcass was recorded in addition to the dry weights of the separate muscles. These values were used to calculate the water content of the carcass and also the percentage of whole body weight represented by the gastrocnemius and thigh muscles. The total fat content of the carcass was determined according to the method of Lundholm *et al* (1980). The dry carcass was crushed in successive 25ml volumes of increasingly volatile extraction mixtures. These mixtures were:

1. Acetone/ethanol (1:1 v/v)
2. Chloroform/methanol (1:1 v/v)
3. Diethylether

The extracts were pooled in a previously weighed round-bottomed flask and the solvents removed using a Buchi rotary evaporator. The weight of the remaining fatty residue was determined and the total fat per carcass calculated.

3.2 In Vitro Characterization of the MAC16 Colon Adenocarcinoma

3.2.1 MAC16 Tissue Culture Cell Line

The MAC16 tissue culture cell line was derived from the *in vivo* MAC16 tumour by Drs. J.A. Double and M. Bibby, Bradford University. The cells were grown in suspension (Ms. J. Cresswell, Aston University) in RPMI 1640 medium containing 5% foetal calf serum with an atmosphere of 95% air/5% CO_2 and had a doubling time of approximately 36 hr.

3.2.2 Sephadex Gel Filtration Chromatography of MAC16 Cells Grown In Vitro

Approximately 1×10^6 cells were harvested and centrifuged at 1000rpm for 5 min in a Heraeus Labofuge 6000 and the supernatant removed. 0.5ml Krebs Buffer was added to the cell pellet and the cells disrupted by sonication on full power for 15-20 sec. The protein content was measured according to the method of Lowry *et al* (1951) as described in Section 3.2.3 and a volume of cell lysate equivalent to 25mg protein was

passed down a Sephadex G50 column previously equilibrated with 10mM potassium phosphate buffer, pH 8.0. The sample was eluted with the same buffer. Fractions (1ml) were collected and assayed for lipolytic activity.

3.2.3 Protein Assay (Method by Lowry et al, 1951)

Reagents were as follows:

Solution A :	2% sodium carbonate in 0.1M sodium hydroxide
Solution B :	1% copper sulphate
Solution C :	2% sodium/potassium tartrate
Solution D :	50ml Solution A + 0.5ml Solution B + 0.5ml Solution C

All solutions were made up to volume with distilled water.

Volumes of the sample (20µl and 50µl) were diluted to 0.4ml with distilled water. Solution D (2ml) was added to each tube and left to stand for 10 min after mixing. Folin and Ciocalteu's Phenol Reagent (0.2ml, diluted 1:1) was added to each tube and the mixtures were left to stand for a further 30 min. A blank sample was included which consisted of 0.4ml distilled water plus reagents. The absorbance was read at 750nm, 25°C on a Beckman DU70 spectrophotometer and the protein content of the samples calculated from a calibration curve using bovine serum albumin as a standard.

3.3 Inhibitors of Cancer Cachexia: An Investigation into the Mechanism of Action of Eicosapentaenoic Acid and BW A4C

3.3.1 Investigation of Eicosapentaenoic Acid as an Inhibibitor of Cancer Cachexia

3.3.1.1 Inhibition of Prostaglandin E2 by Eicosapentaenoic Acid In Vivo

3.3.1.1.1 Extraction of Prostaglandins from Spleens.

Spleens were removed from NMRI mice bearing the MAC16 tumour to which had been fed either EPA (2g/kg/day), DHA (2g/kg/day) or GLA (5g/kg/day) by gastric intubation in addition to a rat and mouse breeding diet. Control mice were administered solvent (liquid paraffin) alone (Dr. S. Beck, Aston University). The spleens were weighed and sliced on filter paper moistened with cold NaCl 0.85%. The slices were incubated for 20 min at 37°C in Krebs Ringer Bicarbonate Solution (2ml) containing 1mg/ml each of BSA and glucose with a gas phase of 95% N_2/5% CO_2. After this time the gas phase was changed to 95% O_2/5% CO_2 and the incubation continued for a further 15 min. The spleen slices were removed at the end of the incubation and the medium frozen immediately at -196°C.

When required for extraction the medium was thawed. A portion (1ml) of this was removed and the pH adjusted to 3 - 3.5 with 2N HCl before extracting twice with ethyl acetate

(saturated with water) (3ml). The extract was evaporated to dryness under a stream of nitrogen and dissolved in 1ml 25mM sodium phosphate buffer, pH 6.8 containing 0.01M EDTA, 0.9% NaCl, 0.3% bovine γ-globulin, 0.005% triton X-100 and 0.05% sodium azide prior to determination of PGE_2 concentration.

3.3.1.1.2 PGE2 Radioimmunoassay

A stock standard solution of 1μg/ml PGE_2 in absolute ethanol was prepared and this was diluted with PG Buffer (Section 2.3.6) to give a range of standard solutions at concentrations from 15-1000pg/0.1ml. Spleen extract or standard solution of PGE_2 (0.1ml) was added to rabbit PGE_2 antiserum reconstituted in PG Buffer (0.5ml). This was mixed and incubated for 30 min at 4°C. [5,6,8,11,12,14,15-^{3}H(N)]PGE_2 as a radioactive tracer (4.26nCi/assay) in PG Buffer (0.1ml) was added and the tubes were vortexed before incubating for 60 min at 4°C. Cold Dextran-Coated Charcoal Suspension (0.2ml) (Section 2.3.7) was added to stop the reaction followed by incubation for 10 min at 0°C in ice/ water. The mixture was then centrifuged at 13,000rpm in a Heraeus Sepatech Biofuge 13 for 15 min at 4°C. The supernatant was transferred to Optiphase Hi-Safe 3 scintillation fluid (10ml) and the amount of radioactivity present was determined using a Packard Tricarb 2000A Liquid Scintillation Analyser.

3.3.1.2 Investigation of the Mechanism of the Anti-Lipolytic Action of Eicosapentaenoic Acid in Cancer Cachexia

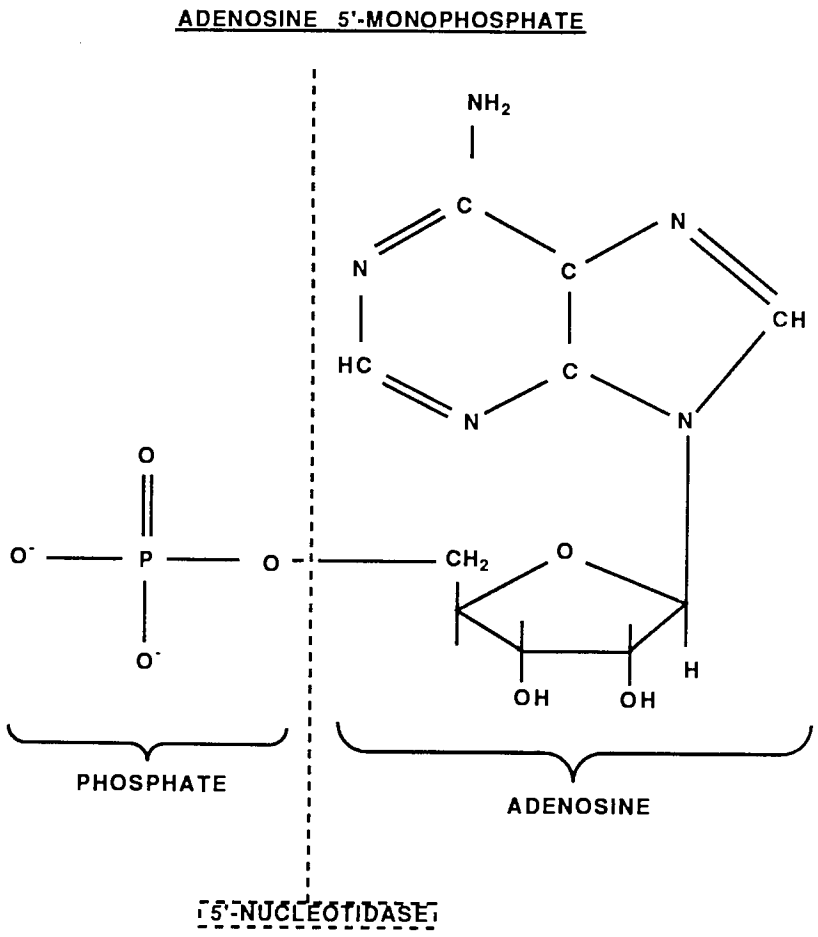
3.3.1.2.1 Preparation of Adipocyte Plasma Membranes

Adipocyte plasma membranes were prepared essentially according to the method of Belsham et al (1980) by means of a self-forming gradient of percoll. Epididymal fat pads (from 500g Wistar rats and 40g BKW mice in experiments using isoprenaline and lipolytic factor respectively) were incubated for 1hr at 37°C in glucose-free Krebs Ringer Bicarbonate solution containing 4% bovine serum albumin and 2mg/ml collagenase. The isolated fat cells were washed three times with Sucrose Buffer (Section 2.3.8) and suspended in approximately 20ml of this buffer. The cells were sheared by rapid aspiration through a Swinny filter. The broken cell suspension was centrifuged at 1000rpm for 5 min in a Heraeus Labofuge 6000. The resulting pellet and infranatant was aspirated from below the fat cake and centrifuged at 30,000g for 30 min at 4°C in an AP Pegasus 65 centrifuge. The supernatant was discarded and the pellet resuspended in Sucrose Buffer (400-500µl). This particulate suspension was added to percoll, Concentrated Sucrose Solution (Section 2.3.9), and Sucrose Buffer (total volume 8ml, mixed in a ratio of 7 : 1 : 32 parts of each component respectively) and centrifuged at 10,000g for 15 min at 4°C. The suspension was divided into fractions approximately 0.75ml in volume using a

Pharmacia Peristaltic Pump P-1 and assayed for various cell fractions. The plasma membrane fraction was washed three times in NaCl Buffer (Section 2.3.10) (centrifuging at 10,000g for 2 min at 4°C each time) before suspending in an appropriate buffer.

3.3.1.2.1.1 5'-Nucleotidase Determination

Figure 3.2



The level of 5'-nucleotidase which is almost

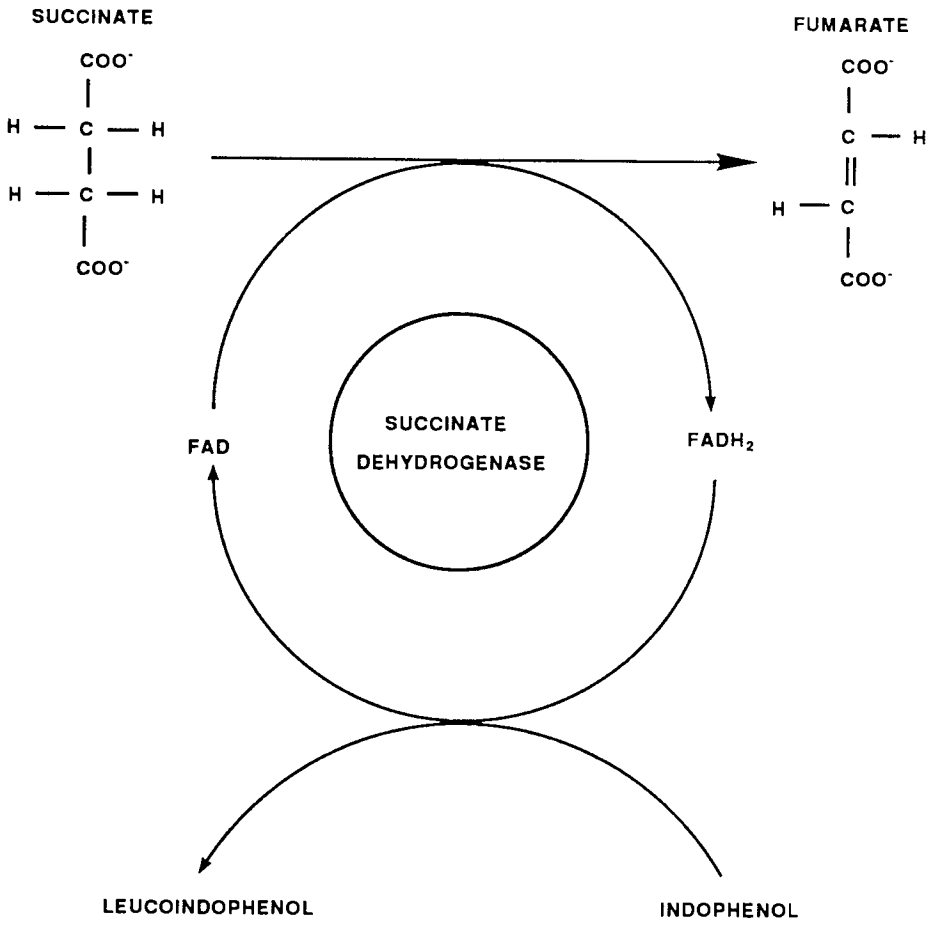
exclusively found in the plasma membrane was measured according to the method of Avruch and Wallach (1970). 5'-Nucleotidase catalyses the hydrolysis of adenosine 5'-monophosphate to adenosine. The sample (50µl) was added to a solution of 50mM Tris, pH 8.0, 180µM $MgCl_2$, and 20µM AMP (sodium salt) with 1µCi [2-^{3}H]AMP (sp. act. 23 Ci/mmol) as a tracer in a final volume of 1ml. The mixture was incubated for 30-60 min at 37°C and the reaction was stopped by the addition of 0.25M $ZnSO_4$ (0.2ml), followed by 0.25M $Ba(OH)_2$ (0.2ml) to precipitate unhydrolysed AMP. This was centrifuged at 13,000rpm in a Heraeus Sepatech Biofuge 13 and the supernatant (0.7ml) was added to 10ml Optiphase Hisafe 3 scintillation fluid. The adenosine remaining in the supernatant was determined by liquid scintillation counting.

3.3.1.2.1.2 Succinate Dehydrogenase Determination

The level of succinate dehydrogenase was determined according to the method of Bachman et al (1966). This enzyme is an integral part of the inner mitochondrial membrane. It supplies electrons to the electron transport chain responsible for generating ATP in the process of oxidative phosphorylation which occurs in the matrix of the mitochondrion. The reaction performed involves the succinate-dependent reduction of indophenol to leucoindophenol and this is measured spectrophotometrically at 600nm. Cuvettes contained 20µl sample and 980µl buffer containing 10mM

potassium phosphate, pH7.4, 0.5mg/ml bovine serum albumin, 2mM KCN, 10mM sodium succinate and 20µg/ml 2,6-dichlorophenolindophenol. The decrease in absorbance was measured for 20 min at 38°C.

Figure 3.3

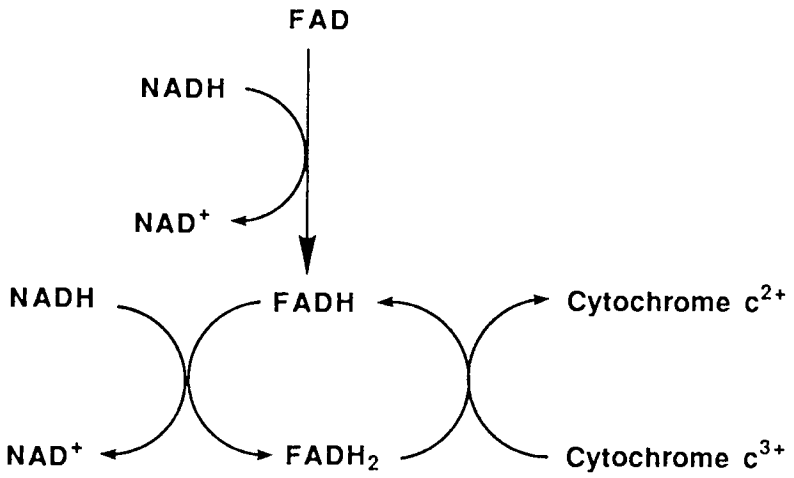


3.3.1.2.1.3 NADH-Cytochrome C Reductase Determination

NADH-cytochrome c reductase is a marker for the endoplasmic reticulum and was measured according to the method

of Dallner et al (1966). The assay is a spectrophotometric one and measures the increase in absorption due to the NADH-dependent reduction of cytochrome c. Cuvettes contained 50µl sample and 2.95ml buffer containing 0.05M potassium phosphate, pH7.5, 0.1mM NADH, 0.33mM KCN and 0.05mM cytochrome c. The absorbance was measured at 550nm for 20 min at 25°C.

Figure 3.4



3.3.1.2.1.4 Lactate Dehydrogenase Determination

Lactate dehydrogenase, a cytoplasmic marker, was measured using the spectrophotometric method described by Keiding et al (1974). This enzyme is responsible for ensuring the continuation of glycolysis under anaerobic conditions by regenerating NAD^+ which is required for the oxidation of glyceraldehyde-3-phosphate in the glycolytic pathway. The assay utilizes the reduction of pyruvate to lactate and

measures the decrease in absorbance due to the formation of NAD^+ from NADH during this reaction. Microfuge tubes containing 25µl sample plus 1ml of a buffer containing 56mM Tris, pH7.4, 5.6mM EDTA, 0.17mM NADH were incubated at 37°C for 10 min. The reaction was initiated by the addition of 100µl of a 13.5mM solution of sodium pyruvate and the increase in absorbance measured at 340nm for 30 min at 37°C.

Figure 3.5

3.3.1.2.1.5 Protein Assay (Method by Bradford, 1976)

Samples were diluted to a volume of 0.8ml with distilled water, added to Bradford reagent (0.2ml) and allowed to stand for 5 min. Samples were calibrated against a control containing water (0.8ml) plus Bradford reagent (0.2ml) and the absorbance read at 595nm at a temperature of 25°C. Protein concentrations were determined from a calibration curve using bovine serum albumin standard solutions.

3.3.1.2.2 Effect of Polyunsaturated Fatty Acids on Pertussis Toxin - Induced ADP-Ribosylation of Adipocyte Inhibitory G-Protein

3.3.1.2.2.1 ADP-Ribosylation Assay

ADP-ribosylation was measured essentially according to the method of Rothenberg and Kahn (1988). Pertussis toxin was pre-activated by incubation at 37°C for 10 min in a freshly prepared solution containing 200mM sodium phosphate, pH7.4, 0.5mM dithiothreitol and 100µM ATP at a concentration of 250µg/ml (Rothenburg et al (1988)). Rat adipocyte plasma membranes were prepared as described in Section 3.3.1.2.1 and 10µl of a suspension containing approximately 75µg protein (measured according to the method of Bradford (1976) as described in Section 3.3.1.2.1.5) in 500mM potassium phosphate, pH7.4 was added to 75µl freshly prepared incubation mixture containing the following substances in quantities to give a final concentration of arginine (10mM), thymidine (10mM), ATP (1mM), $MgCl_2$ (1mM), urea (1mM), potassium phosphate (500mM), pH7.4 in 100µl at 0°C. EPA, DHA, and AA (in equimolar lysine) were added and the suspension incubated for 15 min at 37°C. The incubation was continued for 30 min at 30°C after the rapid addition of activated pertussis toxin (2.5µg), immediately followed by [adenylate-^{32}P]NAD (sp. act. 200 Ci/mmol: 10µCi, final concentration 500nM). The reaction was terminated by the addition of 25mM Tris-HCl, pH7.5 (1ml) at

0°C. The membranes were centrifuged at 12,000g at 4°C for 5 min in an AP Pegasus 65 centrifuge, the supernatant removed and the pellets dissolved in Laemmli Sample Buffer (50µl) (Section 2.3.17). The samples were then heated at 100°C for 5 min and the proteins separated using SDS-polyacrylamide gel electrophoresis.

3.3.1.2.2.2 SDS-Polyacrylamide Gel Electrophoresis

Glass plates (83 ×102mm and 73 × 102mm), spaced 1.5mm apart, were placed in a Biorad Mini Protean II gel system and Running Gel (12%) (Section 2.3.13) poured between them. This was covered with a layer of water and left to polymerize. Stacking Gel (5%) (Section 2.3.15) was poured on top of the Running Gel with the combs in place and left to polymerize. The combs were removed and the wells washed with Electrolyte Buffer (Section 2.3.16) before use. Samples (40µg, measured according to the method of Bradford) were placed directly into the wells and the remaining space filled with Electrolyte Buffer. [^{14}C]Methylated protein molecular weight markers were included on each gel (10µl molecular weight marker plus 190µl Laemmli Sample Buffer (Section 2.3.17), heated as described for samples and 20µl loaded onto gel). The plates were placed in the gel system, this was filled with Electrolyte Buffer and the current was set at 30mA per gel. Gels were stained with Coomassie Blue Gel Stain (Section 2.3.18) for 2 hr and destained (Section 2.3.19) overnight. They were then dried

ready for autoradiographic analysis.

3.3.1.2.2.3 Autoradiography

The dried gel was placed on a sheet of Amersham Hyperfilm MP in an Amersham autoradiography exposure cassette and stored at -70°C for 3 days. The film was developed in Kodak GBX developer and replenisher for 5 min and fixed in Kodak GBX fixer and replenisher for 5 min.

3.3.1.2.3 Determination of Adenylate Cyclase Activity

3.3.1.2.3.1 Adenylate Cyclase Assay

Adenylate cyclase activity was determined essentially using the method of Salomon et al (1974) with an incubation mixture of 25mM Tris, pH7.5, 5mM $MgCl_2$, 8mM creatine phosphate, 16u/ml creatine phosphokinase, 1mM cAMP, 1mM [α-^{32}P]ATP (2µCi, sp. act. 20 mCi/mmol) and GTP (10µM). The reaction was initiated by the addition of adipocyte plasma membranes to give a final reaction volume of 100µl which was incubated at 30°C for 10 min. The reaction was terminated by the addition of 100µl of a stopping solution containing 2% SDS, ATP (40mM), cAMP (1.4mM), pH7.5. [^{3}H]cAMP (1µCi, 50µl) was also added in order to calculate the recovery of cAMP after its isolation. Blanks were included with no plasma membranes present.

3.3.1.2.3.2 Isolation of cAMP

cAMP produced from ATP by adenylate cyclase was isolated according to Method C of Salomon et al (1974). Distilled water (0.8ml) was added to each reaction tube containing the mixture obtained from the adenylate cyclase assay (incubation mixture (100µl), stopping solution (100µl) and [^{3}H]cAMP solution (50µl)). After mixing, the contents of each tube were decanted into columns containing Dowex 50WX8-400 resin (1ml) and the eluate discarded. The eluates from two successive washes with distilled water (1ml) were also discarded. Distilled water (3ml) was then added to each column and the eluate collected in polypropylene test tubes. Imidazole-HCl (1.5M), pH 7.2 (0.2ml) was added to each tube to give a solution of approximate pH 7.5. After mixing, the contents of each tube were decanted into columns containing neutral alumina (0.6g) which had been washed with 0.1M imidazole-HCl, pH 7.5 (8ml). The eluate was collected directly into scintillation vials containing Aquasol (12ml). After the columns had completely drained, 0.1M imidazole-HCl, pH 7.5 (1ml) was added and the eluate collected in the scintillation vials.

A dual count (^{3}H/ ^{32}P) was performed on each sample in a Packard Tricarb 2000A Liquid Scintillation Analyser and the production of [^{32}P]cAMP from [α-^{32}P]ATP calculated.

The Dowex columns were recycled by the addition of 1M HCl (2ml) at the end of each experiment and washing with

distilled water (10ml) on the day of the next experiment.

Recycling of alumina columns was achieved by washing with 0.1M imidazole-HCl, pH 7.5 (8ml) prior to use.

3.3.1.2.4 Investigation of the Interaction of Purified MAC16 Lipolytic Factor with Adenylate Cyclase

3.3.1.2.4.1 Purification of MAC16 Lipolytic Factor

The MAC16 tumour lipolytic factor was purified in three stages. The first stage involved a batch extraction procedure using DEAE cellulose. This was an anion exchange process whereby the factor (from homogenised tumours) was bound to the cellulose and then washed off with 0.3M NaCl. The salt washes were concentrated and freeze dried in preparation for separation using a Q Sepharose column. This is another anion exchange process in which a salt gradient elutes the factor. Active fractions were pooled and freeze dried ready for the final stage of purification. This stage involved a gel filtration procedure using a Superose column. Fractions displaying lipolytic activity were pooled and concentrated, reducing the salt content to $\leq$ 1.5M NaCl. In the majority of cases, purified factor was donated by Miss T. McDevitt and Miss D. Whitter (Aston University).

3.3.1.2.4.2 Dose - Response Relationship for Lipolytic Factor

Purified lipolytic factor was added in concentrations ranging from 0.83 - 6.23 µg to the adenylate cyclase incubation mixture containing adipocyte plasma membranes (100µg) and cAMP production measured as described in Section 3.3.1.2.3.

3.3.1.2.4.3 Time Course For cAMP Production Due To Lipolytic Factor

Lipolytic factor (2.08µg/assay) was utilized to study the time course of its action on adenylate cyclase using adipocyte plasma membranes (100µg). The reaction was stopped at time points from 0-120 min.

3.3.1.2.4.4 Effect of Eicosapentaenoic Acid on Lipolytic Factor - Induced cAMP Production

Adipocyte plasma membranes (100µg), in 25mM Tris, pH 7.5, were incubated with EPA (331µM in equimolar lysine) for 15 min at 37°C before assaying for adenylate cyclase activity using lipolytic factor (2.08µg) to stimulate cAMP production.

3.3.1.2.5 Investigation of the Interaction of Eicosapentaenoic Acid with Adipocyte Adenylate Cyclase and its Link to Adipocyte Lipolysis

3.3.1.2.5.1 Effect of Membrane Protein Content on Adenylate Cyclase Activity

Adipocyte plasma membranes (0-100µg membrane protein) were incubated with isoprenaline (1µM) and the production of cAMP measured as described in Section 3.3.1.2.3.

3.3.1.2.5.2 Effect of Polyunsaturated Fatty Acids on Isoprenaline-Stimulated Adenylate Cyclase Activity.

Adipocyte plasma membranes (100µg, 75µg, 50µg, 25µg or 10µg) were incubated at 37°C in 25mM Tris, pH 7.5 with either eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or arachidonic acid (AA) for 15 min prior to the adenylate cyclase assay. The fatty acids were dissolved in equimolar lysine.

3.3.1.2.5.3 Time Course for Inhibition or Stimulation of Adenylate Cyclase by Eicosapentaenoic Acid

Adipocyte plasma membranes (100µg or 50µg), in 25mM Tris, pH 7.5, were incubated with EPA (331µM in equimolar lysine) for 15 min at 37°C prior to the assay for adenylate cyclase activity. Stimulation of adenylate cyclase was

achieved using isoprenaline (1μM). Samples were removed and the reaction stopped at time points from 1-60 min.

3.3.1.2.5.4 Effect of Varying Isoprenaline Concentration on Inhibition or Stimulation of Adenylate Cyclase by Eicosapentaenoic Acid

Adipocyte plasma membranes (100μg or 50μg), in 25mM Tris, pH 7.5, were incubated with EPA (331μM in equimolar lysine) for 15 min at 37°C and then assayed for adenylate cyclase activity. Adenylate cyclase was stimulated by isoprenaline in concentrations ranging from 0-2.0μM.

3.3.1.2.5.5 Effect of Varying GTP Concentration on Inhibition of Adenylate Cyclase by Eicosapentaenoic Acid and Docosahexaenoic Acid

Adipocyte plasma membranes (50μg), in 25mM Tris, pH 7.5, were incubated with EPA or DHA (both 331μM in equimolar lysine) for 15 min at 37°C. The membrane adenylate cyclase was stimulated by isoprenaline (1μM) in the presence of GTP in concentrations varying from 0-10μM and the resulting production of cAMP measured.

3.3.1.2.5.6 Effect of Polyunsaturated Fatty Acids on Isoprenaline-stimulated Lipolysis in Rat Adipocytes

Epididymal fat pads were removed from male Wistar rats (500g) and adipocytes prepared as described in Section 3.1.1.2. Adipocytes were incubated at a concentration of 1 × 10^5 cells/ml with isoprenaline (1μM) plus EPA, DHA or AA (in ethanol) and the resulting glycerol release measured.

3.3.1.2.5.7 Effect of Pertussis Toxin Treatment on the Inhibition of Adenylate Cyclase by Eicosapentaenoic Acid and Docosahexaenoic Acid

Pertussis toxin was activated as described in Section 2.3.1.2.2.1. Adipocyte plasma membranes (50μg), in 25mM Tris, pH 7.5, were incubated with the activated toxin (25μg/ml), 5μM NAD, 5mM $MgCl_2$, 1mM ATP, 10mM arginine and 10mM thymidine at 30°C for 30 min. This was followed by incubation at 37°C for 15 min with EPA or DHA (both 496μM in equimolar lysine) prior to the assay for adenylate cyclase activity using 1μM isoprenaline to stimulate cyclase activity.

3.3.1.2.5.8 Effect of Eicosapentaenoic Acid and Docosahexaenoic Acid on Isoprenaline-induced Lipolysis in Pertussis Toxin - Treated Adipocytes

Mouse epididymal fat pads were minced in Krebs Buffer

containing collagenase (2mg/ml), gassed and incubated at 37°C for 2 hr as described in the Lipolysis Assay (Section 3.1.1.2). After washing with Krebs Ringer Bicarbonate Solution 1ml fat cells (1×10^5 cells/ml) were aliquoted into microfuge tubes. Pertussis toxin was activated as described in Section 2.3.1.2.2.1 and added to half of the tubes containing fat cells at a concentration of 500ng/ml. The samples were gassed with 95% O_2/5% CO_2 and incubated at 37°C for 90 min. Isoprenaline (1μM), EPA and DHA (both 496μM in ethanol) were added to the appropriate tubes, samples were gassed as before and incubated at 37°C for 90 min. Samples were then prepared for glycerol determination as described in Section 3.1.1.

3.3.1.2.5.9 Effect of Eicosapentaenoic Acid and Docosahexaenoic Acid on cAMP Increase Due to Forskolin

Adipocyte plasma membranes (50μg), in 25mM Tris, pH 7.5, were incubated with EPA or DHA (both 331μM in equimolar lysine) for 15 min at 37°C and cAMP production due to addition of forskolin (25μM, in DMSO) to the incubation mixture was measured.

3.3.1.2.5.10 Effect of Eicosapentaenoic Acid and Docosahexaenoic Acid on Lipolysis Induced by Isoprenaline, Forskolin or Dibutyryl Cyclic AMP

Adipocytes were isolated as described in Section

3.1.1.2 and incubated with isoprenaline (1μM), forskolin (25μM, in DMSO) or dibutyryl cAMP (1mM) plus EPA or DHA (496μM, in ethanol) for 2hr at 37°C. The resulting glycerol release was measured as described previously (Section 3.1.1.3).

3.3.1.2.5.11 Effect of Altering pH of Vehicle for Eicosapentaenoic Acid and Docosahexaenoic Acid on Inhibition of Adenylate Cyclase

Adipocyte plasma membranes (50μg), in 25mM Tris, pH 7.5, were incubated with EPA or DHA (final concentration 248μM or 496μM; fatty acid (5mg) dissolved in lysine 0.1M (1ml)/ Na_2CO_3 0.1M (1ml)) for 15 min at 37°C prior to assay for adenylate cyclase activity. Isoprenaline (1μM) was used to stimulate membrane adenylate cyclase.

3.3.1.2.5.12 Incorporation of Eicosapentaenoic Acid into Adipocyte Plasma Membranes

Adipocyte plasma membranes were prepared from male Wistar rat (500g) epididymal fat pads as described in Section 3.3.1.2.1. Plasma membranes (50μg protein) were dispersed in 25mM Tris, pH7.5 (50μl) and incubated with EPA in concentrations ranging from 0-331μM in equimolar lysine for 15 min at 37°C. The membrane suspensions were then centrifuged at 13,000 rpm in a Heraeus Sepatech Biofuge 13 and the supernatants removed. Membranes and media were each subjected

to a lipid extraction and methylation procedure (Miss E.A. Hudson, Aston University). This method was described by Folch et al, 1957) and a brief outline is as follows. Membranes were sonicated (and media dispersed) in chloroform/methanol (2 : 1, saturated with water) and the chloroform layer extracted twice with methanol/water. An internal standard (margaric acid) and antioxidant were added and the mixture was hydrolysed with 5% NaOH/50% methanol. This was then acidified to remove the sodium salt and the resultant free fatty acid was methylated with boron trifluoride/methanol. The methylated fatty acids were extracted with hexane/chloroform (4 : 1) and the hexane layer evaporated to dryness. The samples were stored at -70°C under argon until required for gas chromatographic analysis (Dr. S. Beck, Aston University).

3.3.1.3 Effect of Eicosapentaenoic Acid on Intracellular Calcium Levels

3.3.1.3.1 HL60 Tissue Culture Cell Line

The HL60 cell line was obtained from human promyelocytic leukaemia cells and has an in vitro doubling time of 24 hr. Cells were resuscitated after removal from the cell bank by rapid heating to 37°C and addition to 10ml RPMI 1640 medium containing 20% foetal calf serum (FCS) at 37°C. The suspension was centrifuged at 1000rpm in a Heraeus Labofuge 6000 for 5 min and the supernatant removed. This was followed

by resuspension in fresh warm medium (as above) in an atmosphere of 95% air/5% CO_2. When cells started to grow, they were passaged in RPMI 1640 medium containing 10% FCS.

3.3.1.3.2 Determination of Cell Viability

1×10^5 cells were centrifuged at 10,000rpm for 30 sec in a Hereaus Sepatech Biofuge 13. The supernatant was removed and the cells resuspended in 5µl of a solution of 0.1% trypan blue dye in sterile phosphate buffered saline. Viable cells were observed using light microscopy and were recognised by their ability to exclude the dye. Non-viable cells appeared blue. A minimum of 300 cells was counted and the percentage of viable cells calculated.

3.3.1.3.3 Determination of Intracellular Calcium.

The method of Thompson et al (1988) was used to determine the intracellular calcium level of differentiated HL60 cells. The cells were differentiated by seeding at a density of 2×10^6 cells/ml in RPMI 1640 medium containing 10% FCS and DMSO at a concentration of 1.3%v/v, and incubating at 37°C for 6 days in an atmosphere of 95% air/5% CO_2. Terminal differentiation was assessed as described in Section 3.3.1.3.4. The cells were collected by centrifugation at 1000rpm in a Heraeus Labofuge 6000 for 5 min and resuspended in the same medium at a concentration of 2.5×10^6 cells/ml with

quin 2-acetoxymethyl ester (2×10^{-5}M, dissolved in DMSO). Cells were gassed with 95% air/5% CO_2 and incubated in universals at 37°C for 1 hr, after which they were harvested and resuspended under the same conditions in the absence of Quin 2-AM. When required 2.5×10^7 cells were harvested, resuspended in Neutrophil Buffer (2ml) and transferred to a $1cm^3$ quartz cuvette.

The resting calcium level was determined by incubating the cells at 37°C for 10 min in a Perkin-Elmer LS-5 Luminescence Spectrometer with continuous stirring and recording the fluorescence value (F). The cells were then lysed by adding 10% Triton X-100 (20µl) and the maximum fluorescence value (F_{max}) recorded. Addition of 250mM EGTA (200µl) gave the value of F_{min} and the following calculation was employed.

$$[Ca^{2+}]\ (nM) = \frac{Kd(F - F_{min})}{F_{max} - F}$$

K_d for Quin 2-AM = 115.

Resting calcium levels below 200nM indicated healthy cells.

Cells were thermally equilibrated for 10 min and f-Met-Leu-Phe (dissolved in DMSO) added in a range of concentrations to determine that which gave the maximum rise in intracellular calcium.

The effect of EPA on f-Met-Leu-Phe - induced intracellular calcium rise was investigated by incubating cells for 10 min at 37°C with either EPA (in ethanol) or ethanol alone in the cuvette before the addition of f-Met-Leu-Phe (10^{-6}M) and monitoring the change in fluorescence over 2 min, taking readings at 5 sec intervals.

3.3.1.3.4 Assessment of Terminal Differentiation

3.3.1.3.4.1 Biochemical Test

This method, described by Langdon and Hickman (1987), determines the percentage of cells which have differentiated to granulocyte-like cells. 1×10^5 HL60 cells were centrifuged at 10,000 rpm for 30 sec in a Heraeus Sepatech Biofuge 13 and the supernatant removed. The cells were resuspended in a 1ml volume of nitrobluetetrazolium (1mg/ml) and 12-O-tetradecanoyl-phorbol-13-acetate (5µg/ml) in phosphate buffered saline and incubated for 30 min at 37°C. The cells were centrifuged and most of the supernatant removed before viewing under a light microscope. Differentiated HL60 cells are stimulated by TPA to produce superoxide which reduces the NBT dye to black formazan granules. A minimum of 300 cells was

counted.

3.3.1.3.4.2 Functional Test

HL60 cells were assessed for terminal differentiation according to their capacity for phagocytosis using the method described by Shaala et al (1979).

Red-dyed yeast particles were obtained from Miss A. Watson (PhD thesis, 1990) and these were complement-coated as follows. Yeast particles (5×10^9) were added to Complement Fixation Buffer (8ml) (Section 2.3.5) and rabbit serum (2ml) and incubated for 30 min at 37°C. The yeast was then washed twice with RPMI 1640 medium and resuspended in medium (20ml). This suspension was stored at -20°C in 0.5ml aliquots until required.

HL60 cells (1×10^5) were centrifuged at 10,000 rpm for 30 sec in a Heraeus Sepatech Biofuge 13 and the cell pellet resuspended in a mixture of RPMI 1640 medium and hepatitis-free AB^+ human plasma (50µl) plus red-dyed complement-coated yeast particles (15µl). The cell suspension was mixed thoroughly and incubated for 1 hr at 37°C. This was followed by centrifugation and the supernatant was removed. Toluidine blue (0.1%) in saline (4µl) was added to counterstain the yeast. Differentiated cells were able to take up the yeast particles by phagocytosis and were viewed using light microscopy. A minimum of 300 cells was counted and the percentage of differentiated cells calculated.

3.3.2 Investigation of BW A4C as an Inhibitor of Cancer Cachexia

3.3.2.1 Effect of BW A4C and Indomethacin on Isoprenaline, ACTH or MAC16 Tumour - Induced Lipolytic Activity

Isolated murine epididymal adipocytes were incubated with isoprenaline (1μM), ACTH (25U) or MAC16 tumour homogenate (100μl) and various concentrations of BW A4C or indomethacin (each dissolved in DMSO) as described in Section 3.1.1.2. Glycerol release was measured as described in Section 3.1.1.3. The effect of BW A4C on basal lipolysis was also investigated.

SECTION 4 : RESULTS AND DISCUSSION

4.1 In Vivo Characterization of the MAC16 Murine Colon Adenocarcinoma

4.1.1 Introduction

In an early study, Costa and Holland (1962) determined that a decrease in body fat occurred after the injection of a non-viable preparation of the Krebs-2-carcinoma, which causes extensive depletion of fat stores in the host. Hollander et al (1987) discovered that a tumour cell line producing a small tumour burden in nude mice caused a depletion of body fat which was almost three times that of a different cell line which produced a large tumour burden. These observations, along with the isolation of several lipid-mobilizing factors from tumour cells (e.g. Masuno et al, 1981; Taylor et al, 1992) suggests that the cachexia associated with malignancy may be, at least in part, related to specific tumour-derived molecules which cause an alteration in the metabolic processes of the host.

Knox et al (1983) noted that patients with long-standing malignancy displayed higher metabolic rates than those with a shorter history of disease. This increase in metabolic rate could be indicative of an increased utilization of fat, which possesses a high calorific value, thus suggesting a direct relationship between loss of body fat and time after initiation of tumour growth. In support of this, Watkin et al (1959) observed a reduction in body fat in cancer patients which was directly proportional to time.

Multiple colon tumours have been induced in NMRI mice by treatment with 1,2-dimethylhydrazine (Double et al, 1975). Several transplantable tumour lines were derived from these, one of which was the MAC16 tumour. This tumour was found to produce a profound cachexia in tumour-bearing animals with no accompanying reduction in food intake (Bibby et al, 1987). Evidence for the production of lipolytic and proteolytic factors by this tumour was put forward by Beck and Tisdale (1987). Animals bearing the MAC16 tumour were found to exhibit higher plasma lipolytic and proteolytic activity than non-tumour-bearing controls and animals bearing the related MAC13 tumour which does not produce cachexia. Beck and Tisdale (1987) have also reported a direct relationship between size of the MAC16 tumour and depletion of host lipid stores.

In the light of the discoveries mentioned above, an attempt has been made to determine the relationship between weight loss, serum lipolytic activity and time after transplantation of the MAC16 adenocarcinoma. A characteristic of the MAC16 tumour is that a small proportion (approximately 15 - 20%) of animals transplanted do not develop cachexia despite normal tumour growth. These animals were referred to as 'non-cachectic' animals and were used as a means of comparison between the tumour-bearing and tumour-induced cachectic states.

4.1.2 Results

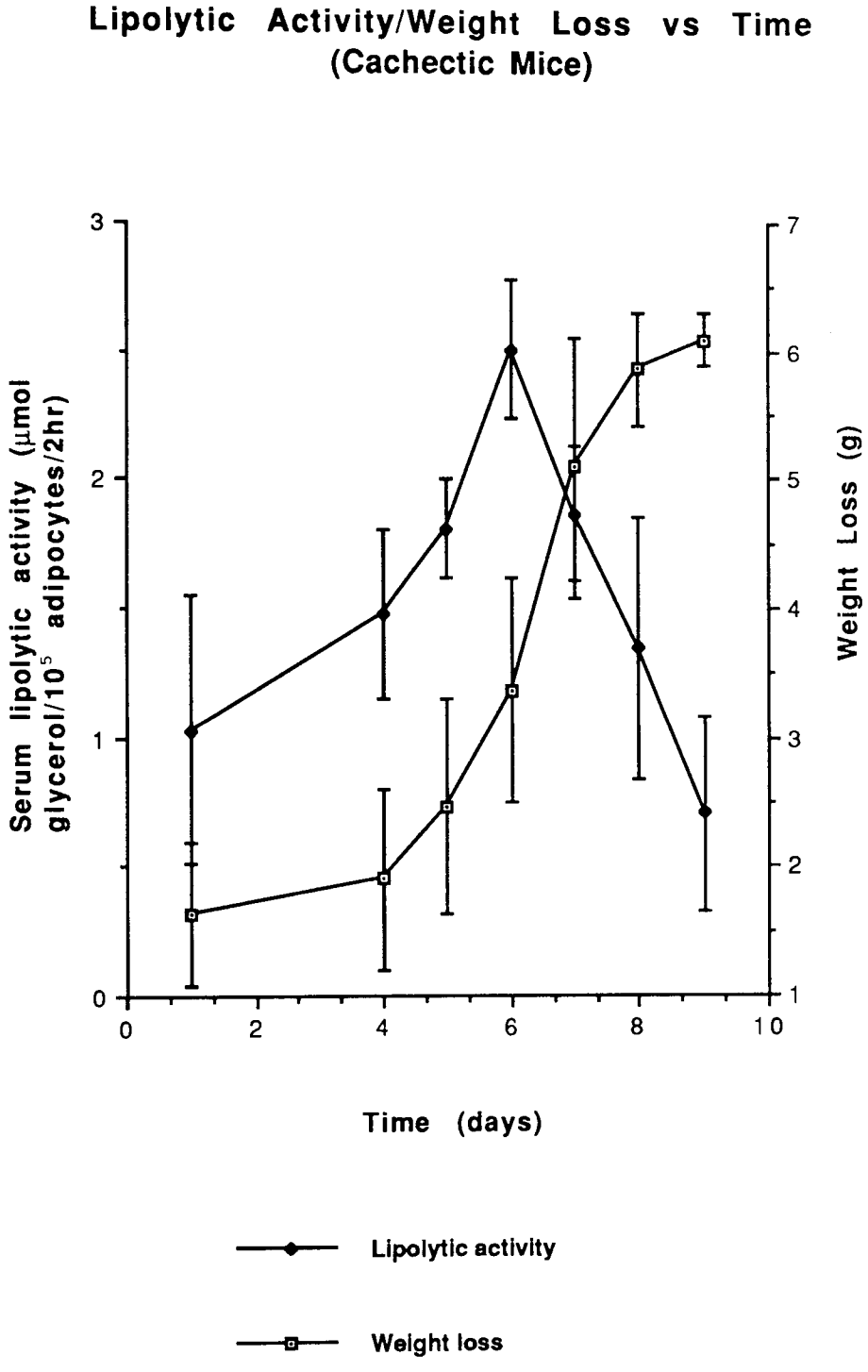
Animals bearing the MAC16 tumour began to lose weight approximately 14 days after transplantation. At this point the cachectic and non-cachectic groups could be differentiated and measurements of serum lipolytic activity were made from both groups. Serum lipolytic activity was expressed per ml serum (5µl sample assayed).

In cachectic animals, the serum lipolytic activity was found to increase in direct proportion to time when the weight loss experienced by the animal did not exceed a value corresponding to 16% of its original body weight (figs. 4.1 and 4.5). After this point an inverse relationship between serum lipolytic activity and time was observed. Values for serum lipolytic activity between days 5 and 7 were significantly higher than the lipolytic activity observed in tumour-bearing, non-cachectic animals and non-tumour-bearing controls (fig. 4.4).

Non-cachectic tumour-bearing animals were found to exhibit serum lipolytic activity which was not significantly different from that seen in non-tumour-bearing animals (fig. 4.2), suggesting that the initial weight loss experienced by MAC16 tumour-bearing animals was directly proportional to an increase in serum lipolytic activity.

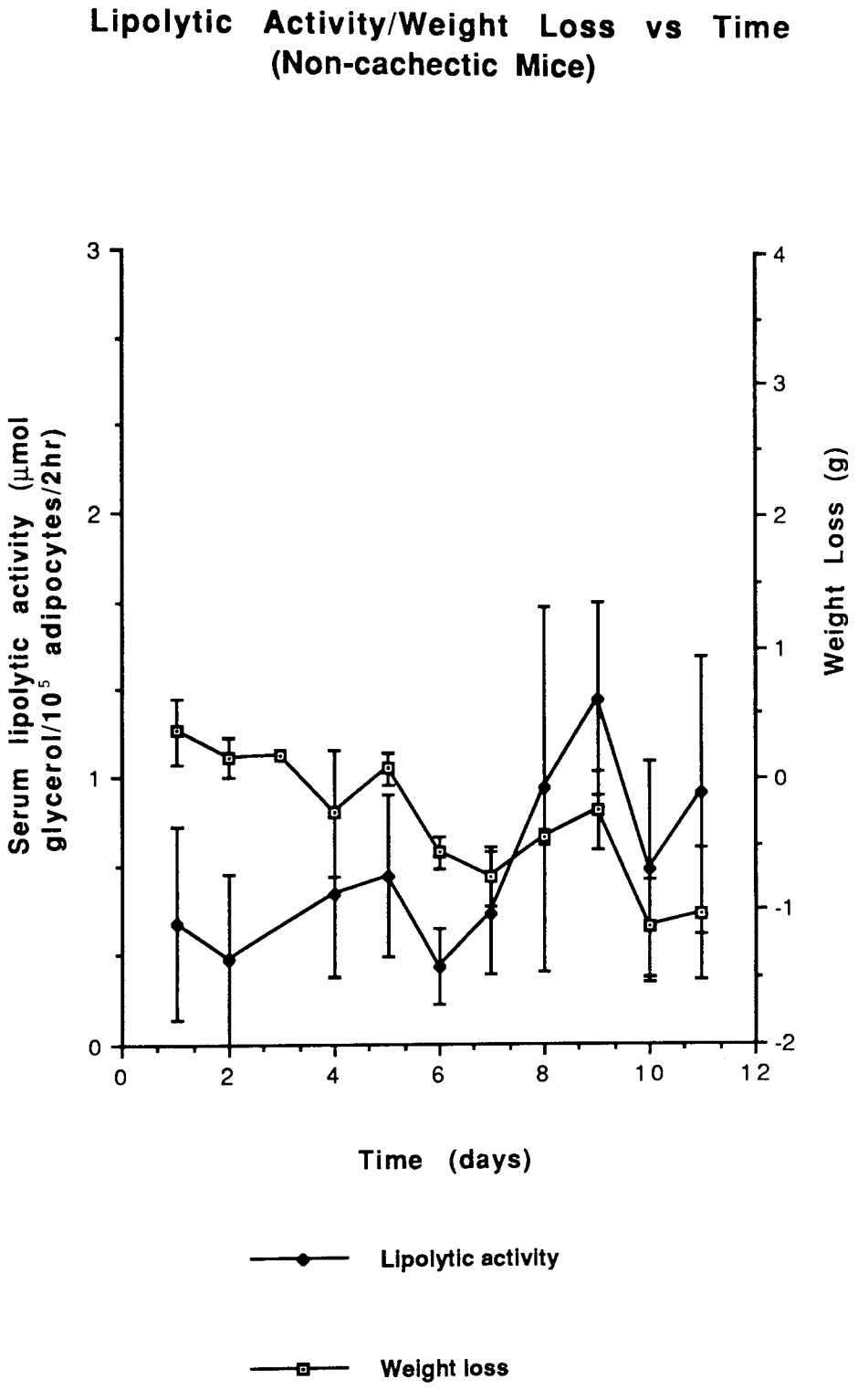
Some animals were found to exhibit a late cachectic response (fig. 4.3). The pattern of weight loss in these animals appeared to follow that of the serum lipolytic

Figure 4.1



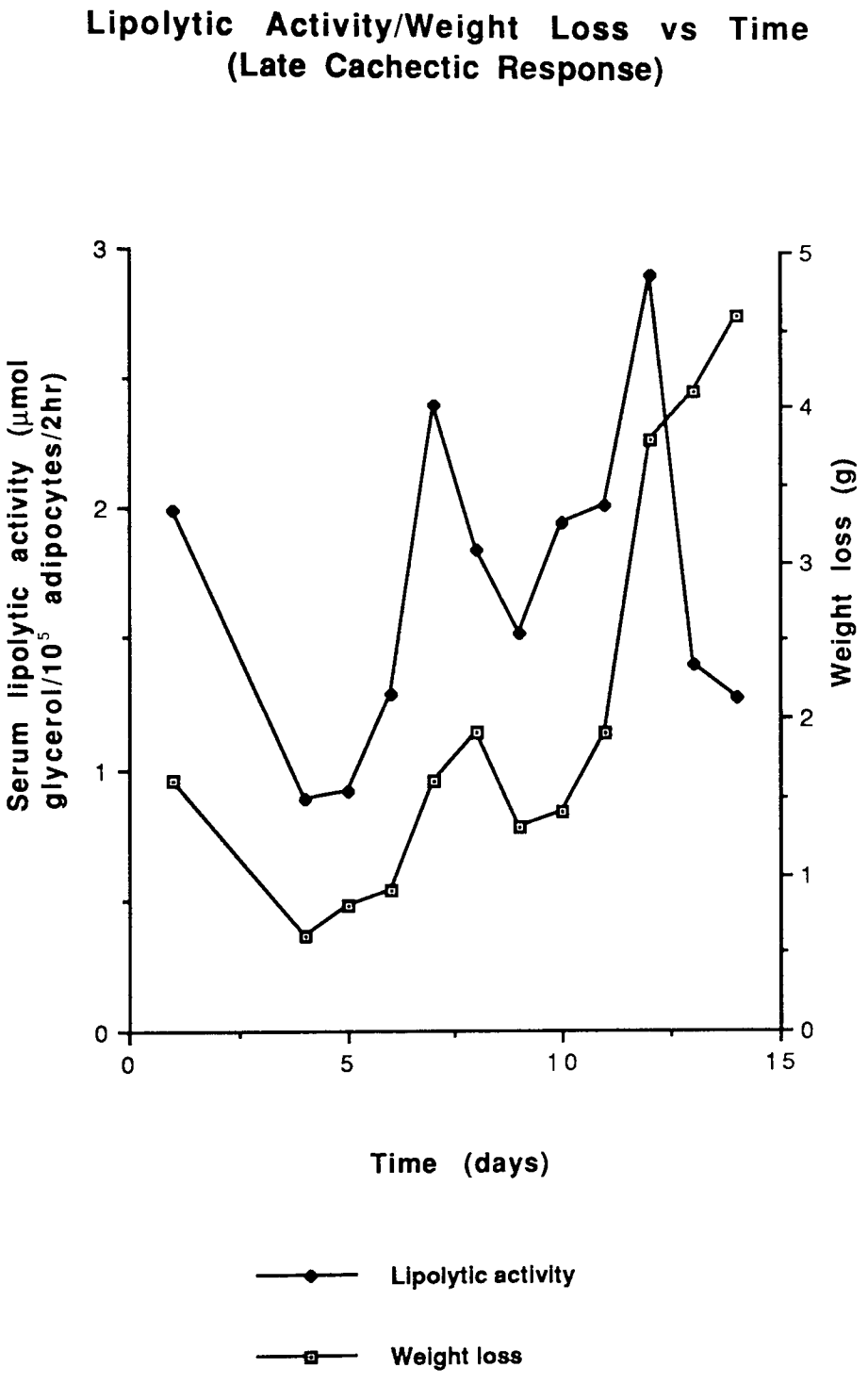
Results are expressed as mean ± SEM of 3 experiments.

Figure 4.2



Results are expressed as mean ± SEM for 4 experiments.

Figure 4.3



Results are from one representative animal.

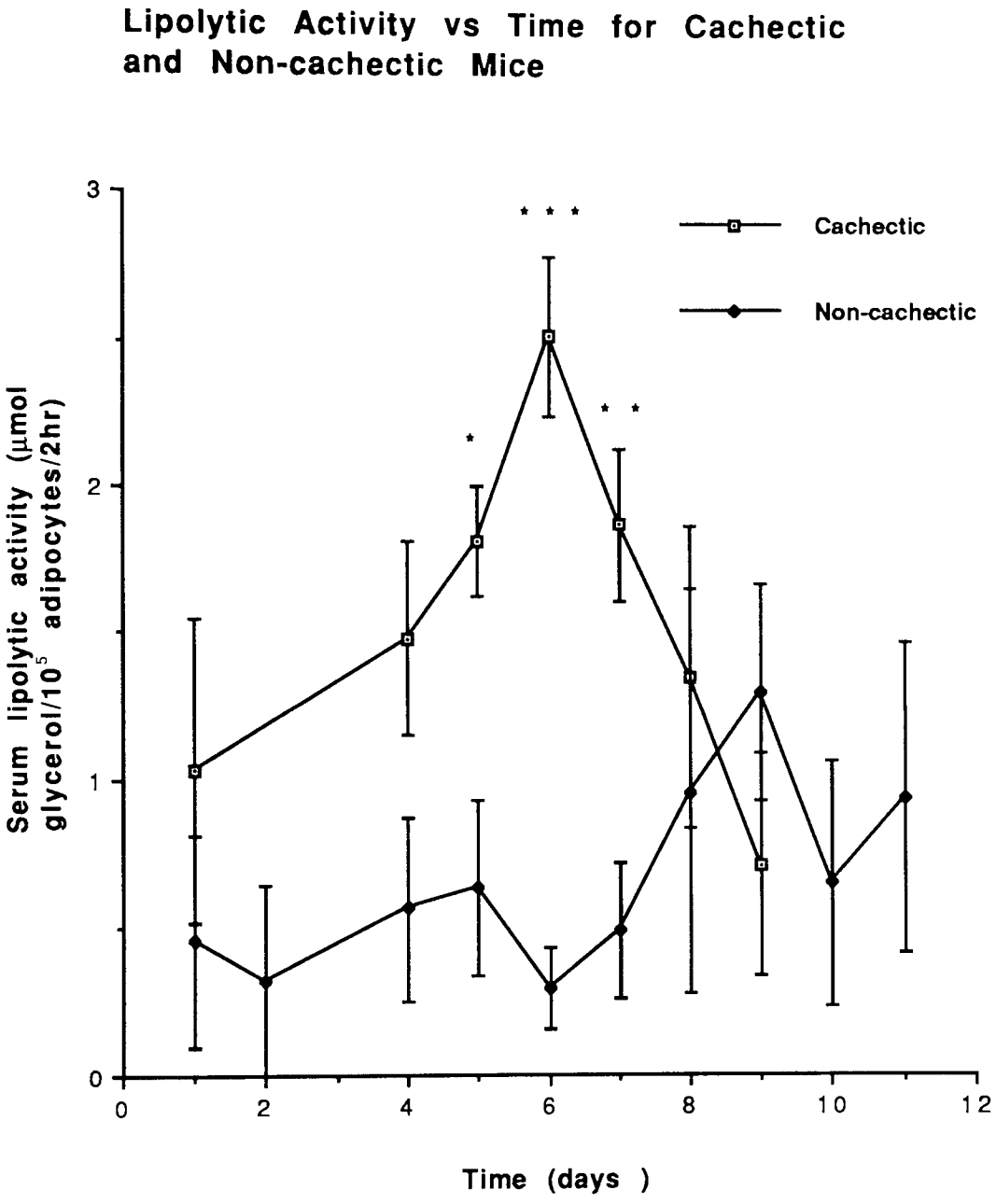
activity.

Body composition analysis of cachectic and non-cachectic animals used in this study showed a large depletion of body fat relative to the reduction in other body compartments in cachectic compared with non-cachectic animals (fig. 4.6 and table 4.1). Loss of body water and non-fat mass did occur in cachectic animals but the reduction in these compartments differed from that of body fat in that it was proportional to the decrease in body weight. This suggests that the weight loss observed in the cachectic animals was primarily due to loss of body fat and that the loss of fat was a direct consequence of the production of a lipolytic factor by the MAC16 tumour.

4.1.3 Discussion

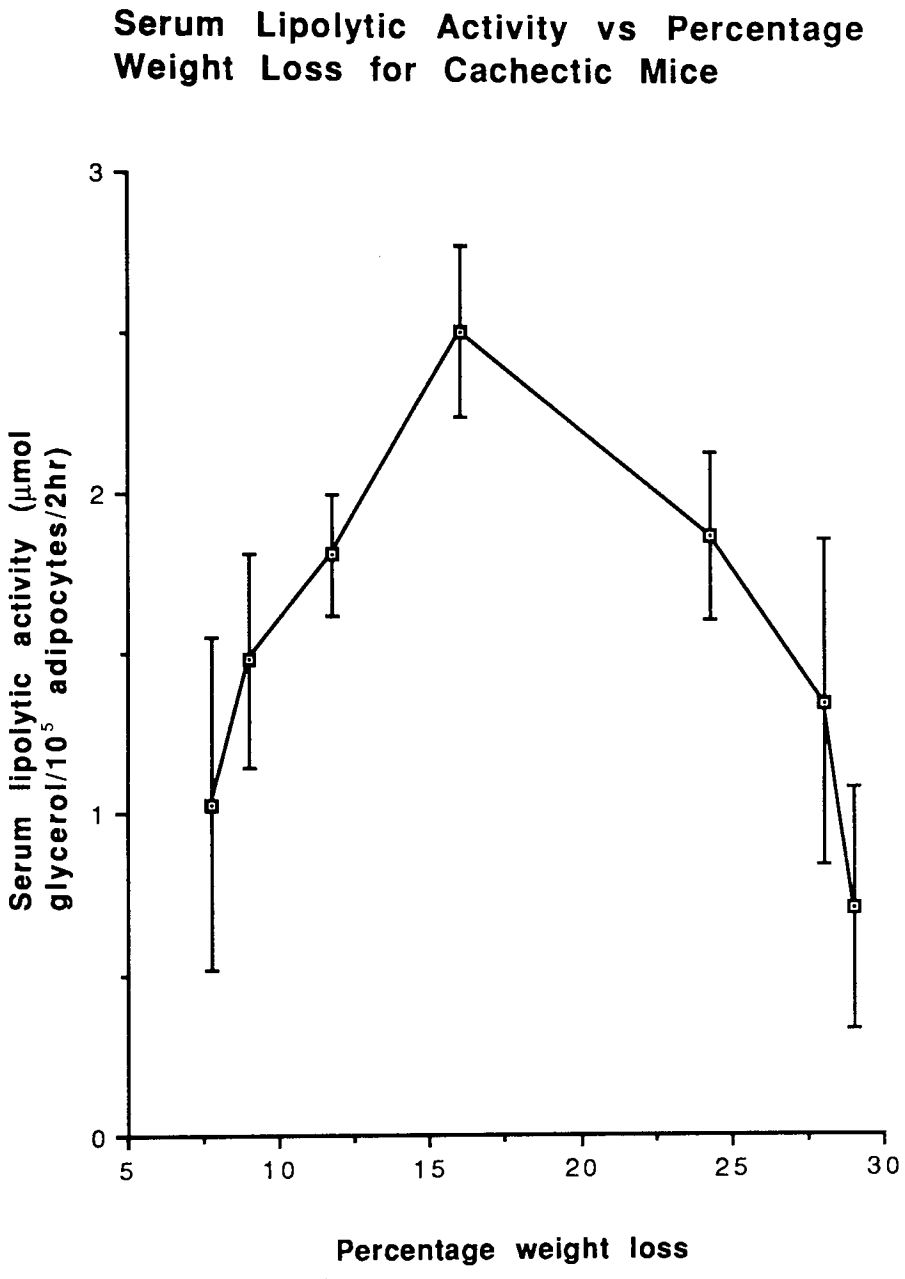
The results obtained with cachectic animals correlated with patient studies (Groundwater et al, 1990) in which the serum lipolytic activity of hospitalized cancer patients was directly related to weight loss below a 20% loss of body weight. Patients with a greater weight loss were found to have low serum lipolytic activity. In these studies, patients with a high percentage loss of body weight who also displayed a low serum lipolytic activity reported a reduced food intake, which suggests that anorexia was probably a more important factor in the continued weight loss at this stage than any circulating catabolic factors.

Figure 4.4



Results are expressed as mean ± SEM for 3 - 4 experiments. Serum lipolytic activity of non-tumour-bearing controls = 0.51 ± 0.18 µmol glycerol/10^5 adipocytes/2hr.
*p < 0.05, **p < 0.01, ***p < 0.001 (cachectic compared with non-cachectic, STT).

Figure 4.5



Results are expressed as mean ± SEM for 3 experiments.

It is not known why the serum lipolytic activity in cachectic MAC16 tumour-bearing animals decreases when the weight loss exceeds 16% of the original body weight. One possible explanation is that the host produces antibodies to the circulating lipolytic factor. Another explanation may be that the lipolytic factor initiates weight loss and its production is switched off when weight loss exceeds 20% of the original body weight. After this point, weight loss becomes a self-perpetuating process as the cachectic subjects become ill. It is possible that the fatty acids released from adipose tissue by the lipolytic factor are responsible for switching off production of the factor since it has been shown that loss of lipolytic activity occurs when MAC16 cells are passaged in vitro in the presence of foetal calf serum which contains fatty acids. A decrease in rate of weight loss of cachectic animals is seen after the peak in serum lipolytic activity, which could be a direct result of the decrease in activity or it could be due to a corrective measure by the host in an attempt to conserve energy.

The importance of lipid availability in cancer is suggested by the fact that an increased rate of removal of infused lipids from the blood of cancer patients was observed by Waterhouse and Nye (1961). A lipolytic factor produced by the tumour in the absence of anorexia would promote lipid mobilization which would not usually occur in the fed state. This factor may therefore provide host tissues with nutrients in a situation where a tumour is utilizing the normal metabolic

Figure 4.6

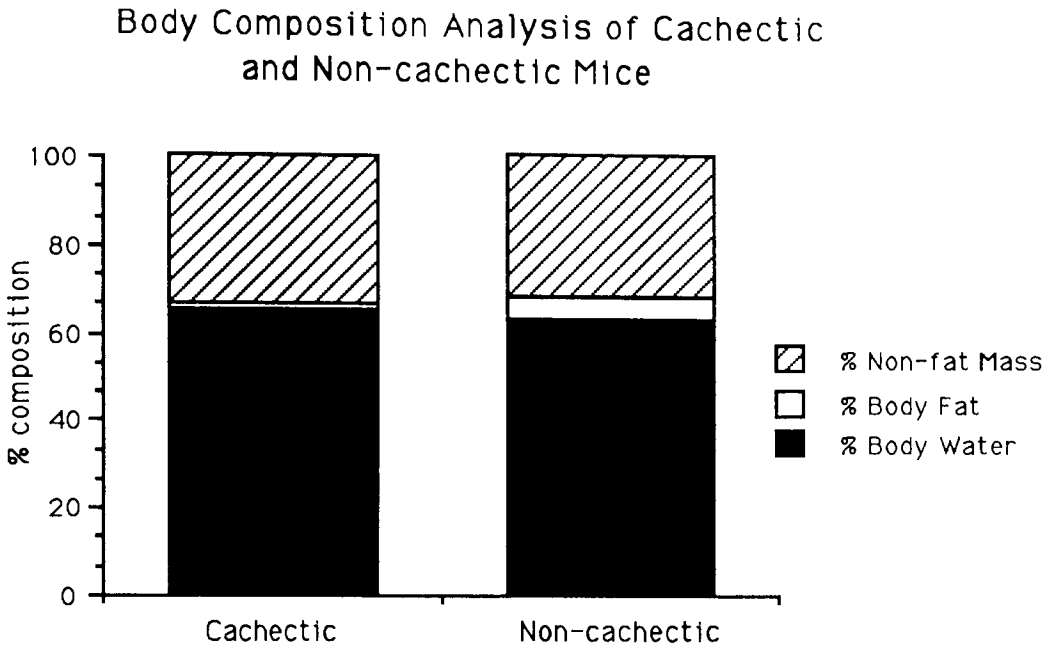


Table 4.1

	Cachectic	Non-cachectic
total weight (g)	13.84 ± 0.42	21.16 ± 0.60
body water (g)	9.12 ± 0.33	13.40 ± 0.21
body fat (g)	0.13 ± 0.03	1.15 ± 0.22
non-fat mass (g)	4.59 ± 0.13	6.60 ± 0.18
gastrocnemius and thigh muscles (g)	0.039 ± 0.003	0.063 ± 0.005
tumour (g)	0.93 ± 0.18	0.69 ± 0.18

Results are expressed as mean ± SEM for 3 experiments.

substrates of the host. Mulligan and Tisdale (1991a) demonstrated that the metabolic processes of the brain, when deprived of glucose by a tumour, are maintained by increased utilization of ketone bodies which are derived from lipid metabolism in the liver. These researchers also noted that increased lipid oxidation occurred in the cachectic, as opposed to the simple tumour-bearing, state (Mulligan et al, 1992). Alternatively, the tumour itself may be utilizing the fatty acids produced by fat mobilization. Although the rapidly-growing MAC16 tumour appears to preferentially utilize glucose as an energy source, with fatty acids released from adipose tissue generally having a regulatory role in the conservation of host tissues (Mulligan and Tisdale, 1991a), the tumour may adapt to utilize fatty acids. This suggestion is supported by the observation that linoleic acid, administered to animals bearing the MAC16 tumour, reverses the anti-tumour effect of eicosapentaenoic acid without altering the anti-cachectic effect (figs. 4.12 and 4.13). Fatty acids released from adipose tissue may therefore provide an alternative energy pathway by which the tumour may support its growth (Beck et al, 1991). The fact that tumour cells appear to possess the ability to utilize host fat metabolites to support their growth was noted by Sauer and Dauchy (1987). Spector (1967) suggested that a significant proportion of the fatty acid required by the Ehrlich ascites tumour is supplied preformed by the host. The lipid was supplied in the form of free fatty acid and mainly incorporated into lipid esters within the

tumour. The fatty acids appeared to arise from mobilization of stores in adipose tissue since incorporation of glucose into tumour lipid was small. Kitada et al (1981) suggested that tumours producing a lipid mobilizing factor utilized the mobilized fatty acids, which would normally provide energy for the host by β-oxidation, for tumour cell membrane synthesis.

The reason for the absence of cachexia in some animals bearing the MAC16 tumour is not known but may be due to the heterogeneity of the tumour. Transplantation of small fragments of tumour may lead to specific lipolytic factor - producing cells within the tumour mass being in a proportion of tumour burden which is insufficient to cause weight loss even with a large tumour mass. Non - lipolytic factor - producing cells may possess a lesser requirement for host lipids. The absence of lipid depletion in these animals was indeed correlated with a low level of serum lipolytic activity. The idea of specific factor - producing cells within the tumour was further substantiated by the observation that some of the animals initially deemed to be non-cachectic began to lose weight at a later stage in the experiment (fig. 4.3). This was supposedly at a time when growth of the tumour had reached a stage where lipolytic factor production was at a level which was sufficient to cause loss of body weight. The weight loss appeared to follow the pattern of lipolytic activity in the serum of these animals.

It has been suggested that tumour necrosis factor

(TNF) is the cachexia - inducing factor in malignant disease. However, TNF produces an anorectic response in NMRI mice and has no direct effect on adipocytes under the conditions stated in this experiment (Mahony et al, 1988). Consequently, since the MAC16 tumour produces no anorectic response and the factor it produces shows lipolytic activity in vitro it can be concluded that the factor causing the weight loss in this experiment is not TNF.

The conclusion of this study is that some tumours produce a factor which exerts a direct lipid - mobilizing effect on adipose tissue. The weight loss produced by this factor appears to be directly related to the level of lipolytic activity detectable in the serum of tumour-bearing subjects in the initial stages of disease, and this observation may provide a useful diagnostic tool for cancer when more is known about the structure of this factor.

4.2 In Vitro Characterization of the MAC16 Murine Colon Adenocarcinoma

4.2.1 Introduction

The lipolytic factor, toxohormone L, isolated by Masuno et al (1981) is a 75 kDa protein. More recently a 6000 Da lipolysis-promoting factor has been isolated by Taylor et al (1992) from the media in which the human A375 melanoma cell line was grown. This factor appears to be similar to that produced by the MAC16 tumour in that it produces weight loss which is correlated with a decrease in carcass fat and exerts a direct lipolytic response in isolated adipocytes.

Supernatant obtained by centrifugation of a homogenate of the MAC16 tumour (Beck, 1989) produced three peaks of lipolytic activity when analysed using Sephadex gel filtration chromatography which utilizes the difference in size of molecules which are to be separated. A Sephadex G50 column showed activity corresponding to approximate molecular weights 3.0, 1.5 and 0.7 kDa. The same three peaks were obtained with serum and tumour samples from cancer patients (with or without weight loss) but were not detectable in serum from cachectic patients with Alzheimer's disease, burns or in controls in the fed or starved state. It was not known at this stage whether these tumour-associated factors were produced by the tumour cells or by an indirect action of the tumour which induced the breakdown of an endogenous lipolytic hormone.

This study utilized the MAC16 tissue culture cell line in order to determine whether the MAC16 lipolytic factors isolated by Beck (1989) are products of tumour cells. A Sephadex G50 column was used to separate fractions of a cell lysate obtained from this cell line.

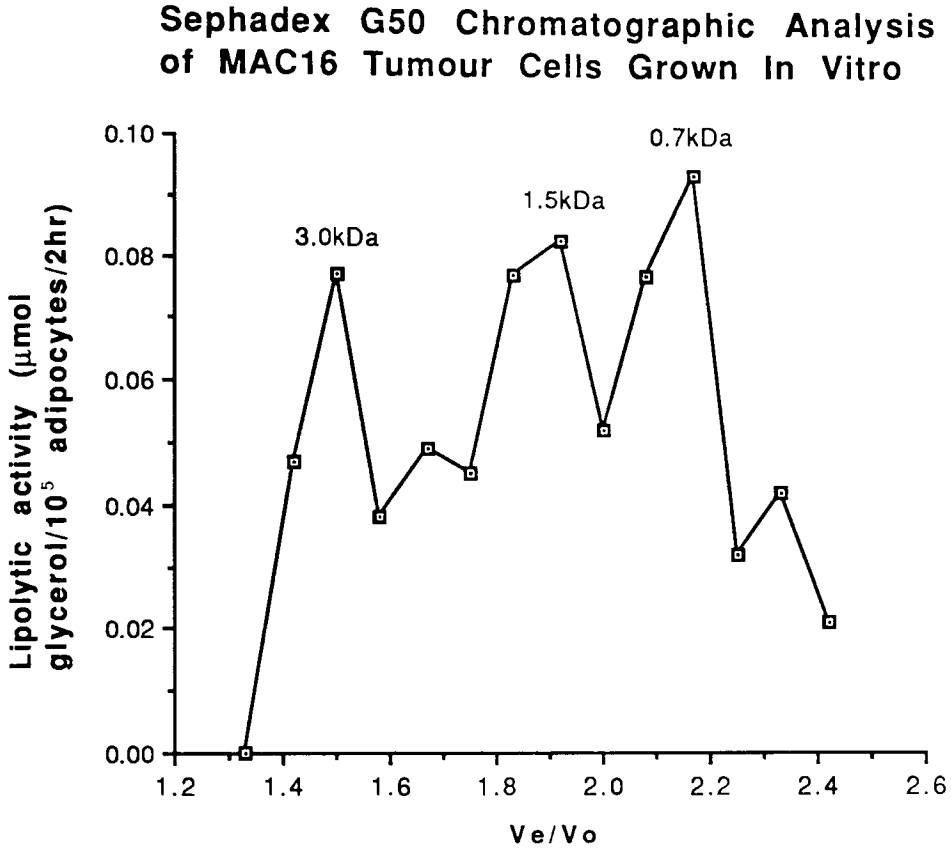
4.2.2 Results

The results presented in fig. 4.7 show the appearance of three active fractions from the MAC16 cell lysate with molecular weights corresponding to the three peaks obtained from tumour, serum and urine samples by Beck (1989).

4.2.3 Discussion

The results confirmed that the MAC16 tumour produces more than one lipolytic factor, or that a single lipolytic factor may be cleaved into smaller molecules which retain this activity *in vitro*. The observation that lipolytic substances produced from tumour cells grown *in vitro* have molecular weights comparable to factors present in samples obtained from tumour-bearing hosts suggests that the factor(s) are products of the tumour cells and are not endogenous substances secreted in response to a tumour. This observation also further distinguishes the MAC16 lipolytic factor from tumour necrosis factor which is produced by macrophages associated with and in response to a tumour.

Figure 4.7



V_e = elution volume

V_o = void volume

Protein content of sample = 25mg/ml.

Peaks correspond to molecular weights determined from calibration curve (S. Beck) using:

Rifampicin	834Da
Actinomycin D	1247Da
Aprotinin	6600Da
Cytochrome c	12400Da

4.3 Inhibitors of Cancer Cachexia: An Investigation into the Mechanism of Action of the ω-3 Fatty Acid, Eicosapentaenoic Acid, and the 5-Lipoxygenase Inhibitor, BW A4C

The discovery and characterization of a tumour product which exerts a direct lipolytic action on host adipose tissue has directed the search for anti-cachectic agents towards substances which specifically inhibit lipolysis by direct action on the adipocyte. Two agents, eicosapentaenoic acid (EPA) and N-(3-phenoxycinnamyl)acetohydroxamic acid (BW A4C), which have demonstrated anti-tumour and anti-cachectic action *in vivo* (Tisdale and Dhesi, 1990; Beck, S.A. and Hudson, E.A., unpublished results) have been further investigated in order to elucidate the mechanism of their anti-cachectic effect.

4.3.1 Investigation of Eicosapentaenoic Acid as an Inhibitor of Cancer Cachexia

Research into the relationship between dietary fat and incidence of cancer has aroused much interest in recent years. Much of the research has centred around the polyunsaturated essential fatty acids of the ω-6 series which are derived largely from vegetable oils and those of the ω-3 series from fish oils. The categorization of these fatty acids arises from the position of the nearest double bond to the terminal methyl group (containing the ω carbon atom).

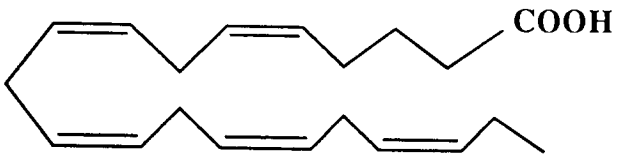
Structures of the ω-6 polyunsaturated fatty acids (PUFAs), arachidonic acid and γ-linolenic acid and the ω-3 PUFAs, eicosapentaenoic acid and docosahexaenoic acid are shown in fig. 4.8. Since chain elongation and desaturation, which is a common occurrence after ingestion of these fatty acids, occurs only at the terminal carboxyl group (containing the α carbon atom), all ω-6 fatty acids are biochemically interrelated, as are all ω-3 fatty acids (fig. 4.9), but no interconversion can take place between the two series (Willis, 1987).

The role of the ω-3 essential fatty acids has been of particular interest following the observation of a low incidence of cancer in Alaskan and Greenland Eskimos, who consume a large amount of fish, compared to that seen in Western populations (Waterhouse et al, 1976).

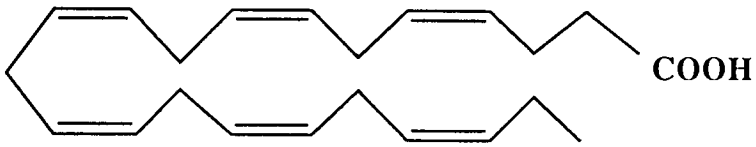
Begin et al (1986) reported the selective killing of tumour cells and reduction in the rate of division of normal cells in vitro by ω-6 and ω-3 fatty acids. The sensitivity of tumour cells to fatty acids varied greatly according to the cell line and cell density used. Most of the studies investigating the anti-tumour effect of ω-6 and ω-3 fatty acids have suggested the role of the ω-3 series to specifically inhibit tumour growth, in contrast to the ω-6 series which appeared to stimulate tumour growth in some cases. Reddy and Maruyama (1986) reported a decrease in the incidence and multiplicity of azoxymethane-induced tumours in rats which were fed a diet supplemented with 4% or 22.5% Menhaden oil (rich in ω-3 fatty acids) plus 1% corn oil (rich in ω-6 fatty

Figure 4.8

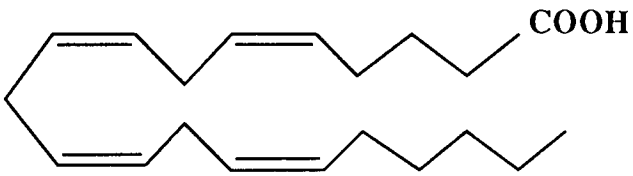
EICOSAPENTAENOIC ACID



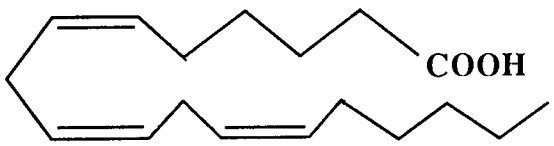
DOCOSAHEXAENOIC ACID



ARACHIDONIC ACID

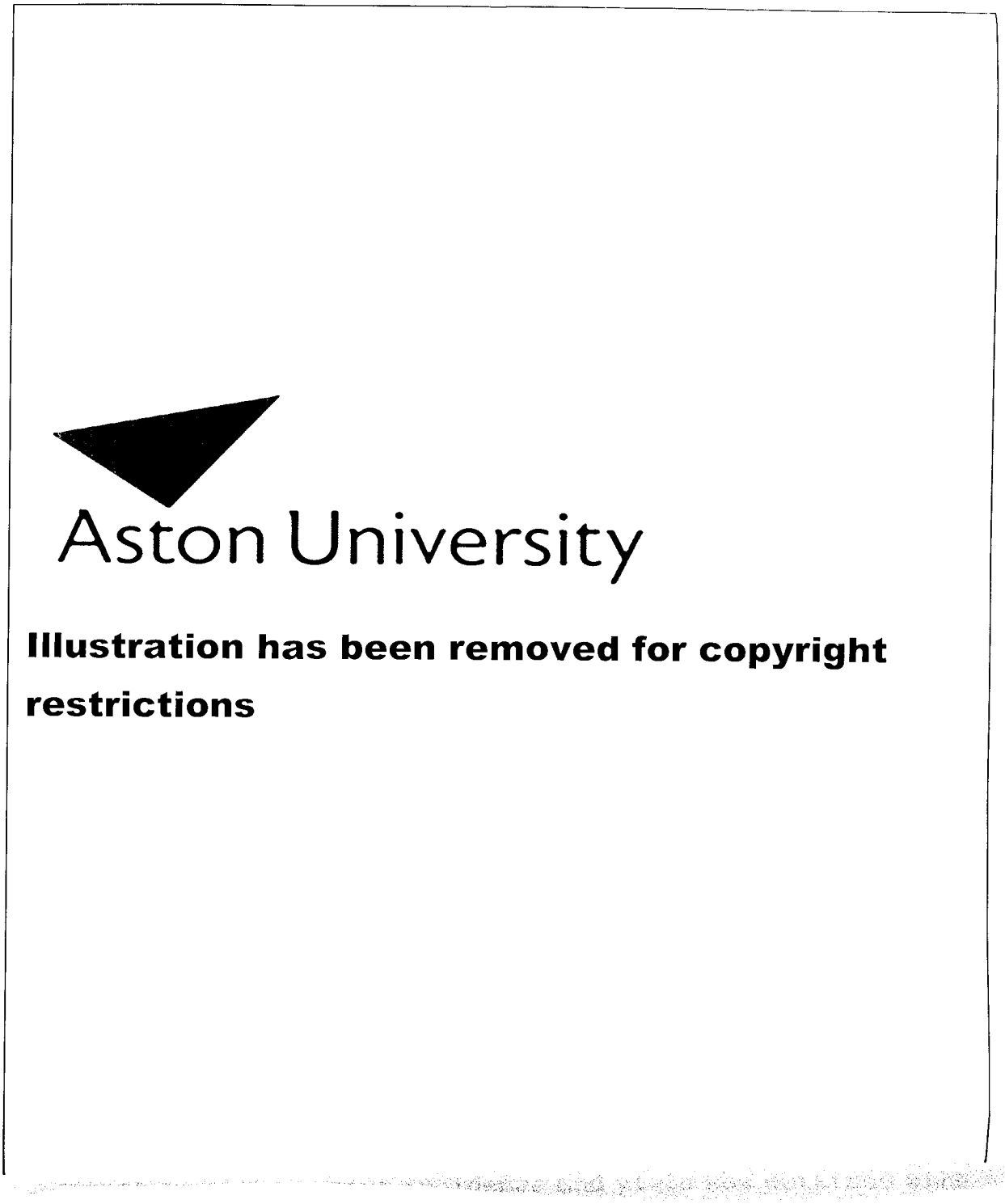


GAMMA LINOLENIC ACID



Structure of Polyunsaturated Fatty Acids

Figure 4.9



Interconversion of Polyunsaturated Fatty Acids and Formation of Eicosanoids

Karmali, 1987

acids) compared with a diet supplemented with 23.5% corn oil. A similar experiment showed that increasing levels of Menhaden oil in the diet were associated with significantly increased levels of eicosapentaenoic and docosahexaenoic acids (the major constituent fatty acids (ω-3) of fish oil) in microsomal fractions from colonic mucosa and tumour tissue (Reddy and Sugie, 1988). Levels of linoleic, linolenic and arachidonic acids (ω-6) were decreased in these fractions suggesting competition by ω-3 with ω-6 fatty acids for incorporation into host and tumour tissue. This relative deficiency was suggested by Carroll (1989) as one explanation for the anti-tumour effect of ω-3 fatty acids. Indeed, the ω-6 fatty acid, linoleic acid, has been found to increase tumour incidence and total tumour burden in a dose-related but saturable fashion when 7,12-dimethylbenz(α)anthracene was used to induce mammary tumorigenesis (Ip, 1987). Wicha et al (1979) reported a doubling of growth rate of malignant and normal cells in vitro by linoleic acid. Sauer and Dauchy (1988) observed increased tumour growth and [^{3}H]thymidine incorporation in starved rats which was attributed to the mobilization of fat stores. Linoleic and arachidonic acids were found to be the limiting factors on tumour growth of this hyperlipidaemic state.

In a study by Minoura et al (1988), a significant decrease in colon tumour incidence and yield was obtained with a diet enriched with EPA when compared with a linoleic acid - rich diet. A similar result was obtained in a recent study by Sakaguchi et al (1990). They observed a reduction in the growth

rate of two human colonic cell lines transplanted into nude mice when the mice were fed MaxEPA and compared with mice fed coconut oil (high in saturated fats). Significant incorporation of ω-3 fatty acids into erythrocyte membranes, adipose tissue and tumour lipid fractions was noted with an accompanied reduction in linoleic and arachidonic acids in these tissues.

4.3.1.1 Investigation of the Relationship Between Eicosapentaenoic Acid and Prostaglandins in Cancer Cachexia

4.3.1.1.1 Introduction

Karmali (1987) suggested that the competition of ω-3 with ω-6 fatty acids may lead to an alteration in eicosanoid production and metabolism. Culp et al (1979) suggested that eicosapentaenoic acid (EPA) competes directly for cyclooxygenase and reduces the excessive prostaglandin E_2 synthesis associated with some tumours. EPA is a substrate for cyclooxygenase and is a precursor of the 3-series of prostaglandins, the biological function of which is unknown. Docosahexaenoic acid (DHA) is not a precursor of 3-series prostaglandins, but is a strong competitive inhibitor of prostaglandin synthetase, thus inhibiting arachidonic acid metabolism and the synthesis of 2-series prostaglandins (Corey et al, 1983).

Karmali et al (1984) observed a decrease in the

synthesis of 2-series prostaglandins in tumour tissue with a corresponding inhibition of tumour growth in rats fed MaxEPA (a fish oil concentrate, particularly rich in EPA). Similarly, Tashjian et al (1984) reported that treatment of mice with Menhaden oil led to decreased levels of prostaglandins in tumour tissue and prevention of the elevation in plasma prostaglandin levels seen in control tumour-bearing animals.

The relevance of the increased level of prostaglandins observed in some tumours is not fully known. One theory is that PGE_2 and cAMP (which is increased by PGE_2 in most tissues) affect cellular proliferation and differentiation in addition to the dissemination of tumours (Droller, 1981). In addition to the animal models mentioned above, excessive amounts of prostaglandin have been found in human tumours (Bennett, 1977).

The strong suggestions of a link between tumour growth and prostaglandin production led to this preliminary study of the mechanism of action of EPA. The MAC16 tumour was utilized as a model of cachexia (see Section 4.1) and the prostaglandin level in the spleens of tumour-bearing mice treated with either EPA, DHA or GLA (γ-linolenic acid) was measured. The object of the study was to determine whether a reduction in prostaglandin levels could account for a reduction in tumour growth and consequently a reversal of the cachexia induced by that tumour.

4.3.1.1.2 Results

The results presented in figs. 4.10 and 4.11 show the effect of treatment with EPA, DHA and GLA on the production of PGE_2 by the spleens of cachectic mice bearing the MAC16 tumour. A decrease in prostaglandin levels was observed in the spleens of mice treated with EPA (2g/kg) and DHA (2g/kg). This decrease was significant for EPA ($p < 0.01$) in one of the two experiments conducted with this fatty acid but not in the other ($p < 0.1$). The decrease in prostaglandin production observed in the spleens of cachectic mice treated with DHA was not significant ($p < 0.1$) compared with solvent-treated controls. A slight increase, which was not significant, was observed in the levels of PGE_2 in the spleens of mice treated with GLA (5g/kg).

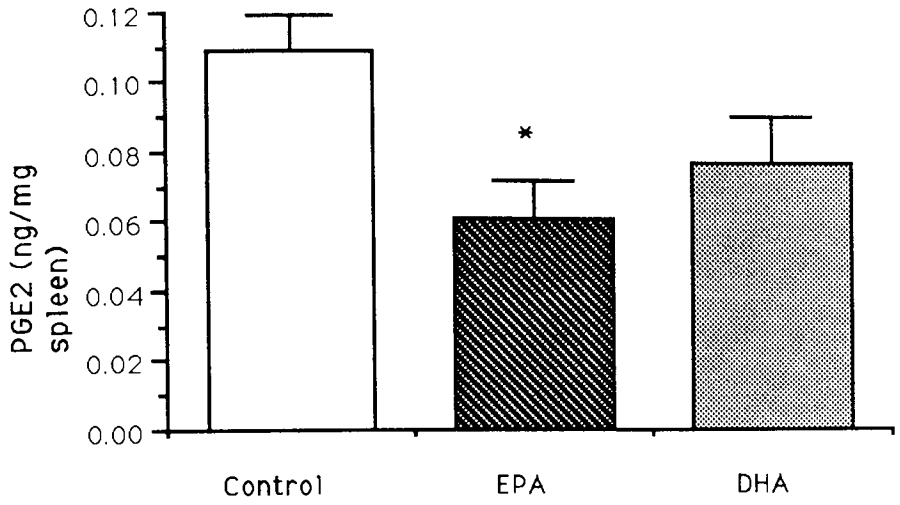
4.3.1.1.3 Discussion

A fish oil diet inhibited growth of the MAC16 tumour with an anti-tumour effect comparable to that induced by cyclophosphamide or 5-fluorouracil (Tisdale and Dhesi, 1990). An additional reduction in the weight loss experienced by animals bearing this tumour was seen with the fish oil diet but not with the two conventional cytotoxic drugs. A diet rich in γ-linolenic acid (GLA) was ineffective in both aspects.

Eicosapentaenoic acid has been found to inhibit weight loss and growth of the MAC16 tumour in tumour-bearing

Figure 4.10

Effect of Eicosapentaenoic and Docosahexaenoic Acids on Prostaglandin Production by Spleen

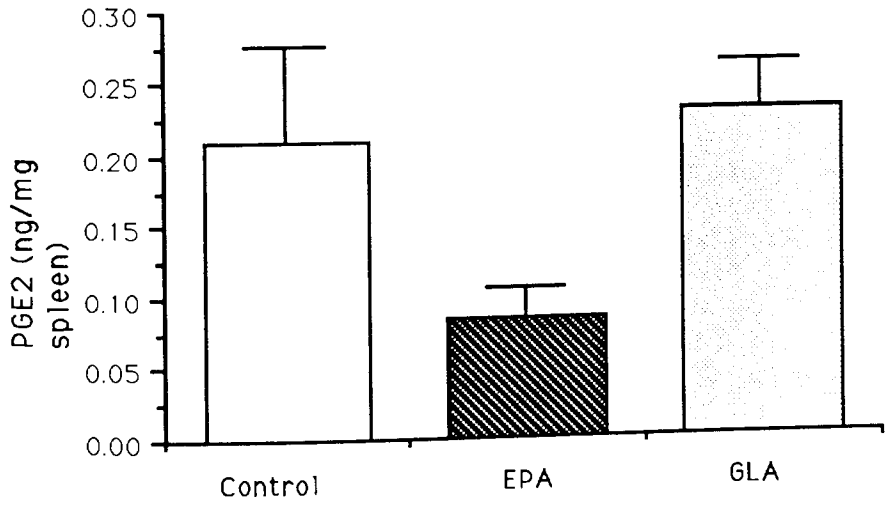


Results are expressed as mean ± SEM of 4 experiments.

*p < 0.01 (compared with controls, STT).

Figure 4.11

Effect of Eicosapentaenoic and Gamma Linolenic Acids on Prostaglandin Production by Spleen



Results are expressed as mean ± SEM of 4 experiments.

animals (Beck, S.A. and Hudson, E.A., unpublished results) (figs. 4.12 and 4.13). The other ω-3 fatty acid constituent of fish oil, docosahexaenoic acid, was found to be ineffective (figs. 4.14 and 4.15). GLA, which is a precursor for 1-series prostaglandins, showed a slight increase in PGE_2 production in this study. A study by Beck et al (1991) demonstrated a slight anti-cachectic/anti-tumour (fig. 4.16) effect due to GLA in high doses. This was probably due to the formation of PGE_1 which reduces cAMP levels in adipocytes leading to a decrease in lipolysis. The object of this experiment was therefore to determine any difference between EPA and DHA on prostaglandin production since EPA, but not DHA, possesses anti-tumour/anti-cachectic activity. Since both EPA and DHA inhibit prostaglandin production in tumour-bearing mice to a similar extent it is unlikely that EPA produces a reduction in tumour growth due to a decrease in prostaglandin levels.

It has been noted that the inhibitory effect of EPA on weight loss is greater than its effect on tumour growth (Beck et al, 1991). This result, in addition to the observation that linoleic acid reverses the anti-tumour (fig. 4.13), but not the anti-cachectic (fig. 4.12), effect of EPA in animals bearing the MAC16 tumour, suggests that two distinct mechanisms may exist for the inhibition of weight loss and tumour growth by EPA and/or that inhibition of the cachectic process has a direct consequence on tumour growth by depriving it of an essential nutrient (Beck et al, 1991). The results of the present experiment failed to show a mechanism by which EPA

Figure 4.12

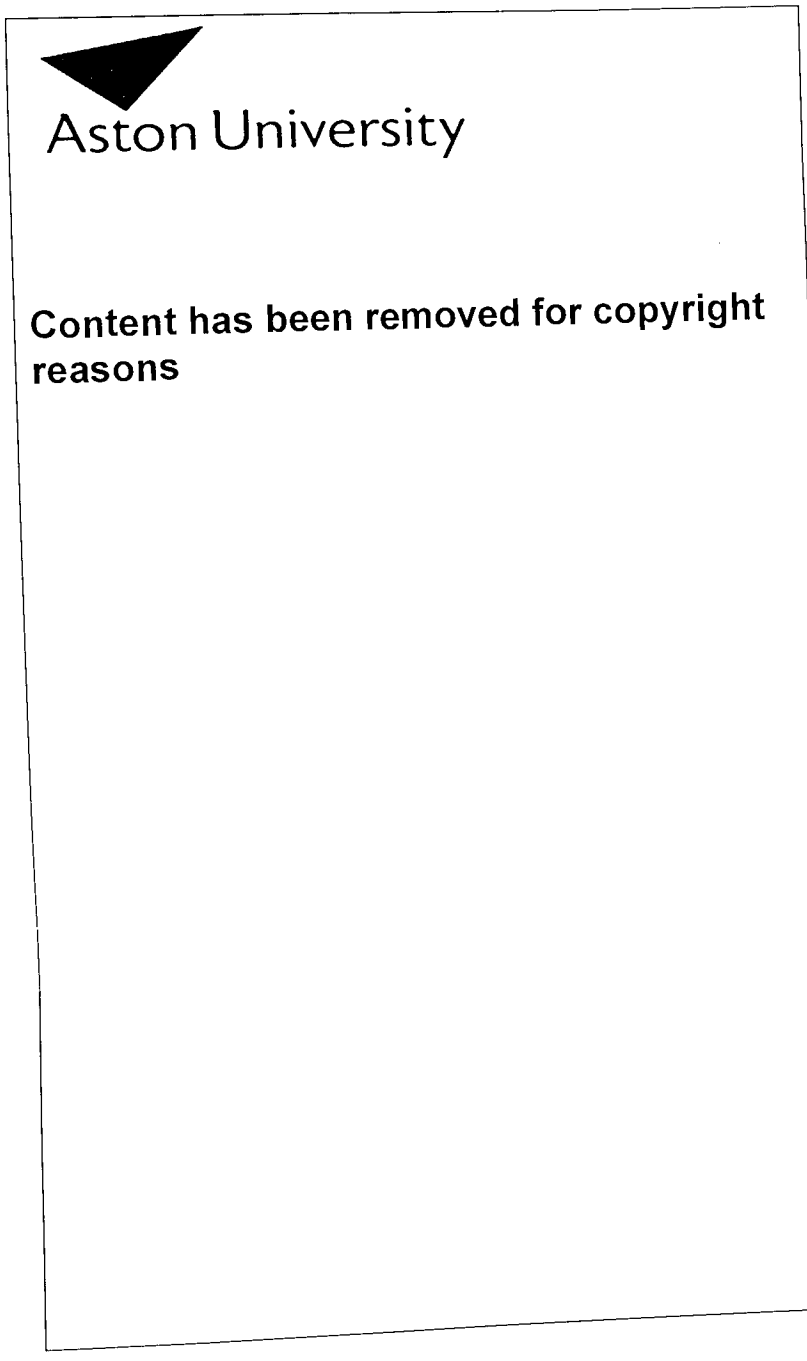
Effect of EPA and/or LA on Body Weight

Anti-Cachectic Effect of Eicosapentaenoic Acid

Beck, S.A. and Hudson, E.A.

Figure 4.13

Effect of EPA and/or LA on Tumour Volume



Anti-Tumour Effect of Eicosapentaenoic Acid

Beck, S.A. and Hudson, E.A.

Figure 4.14

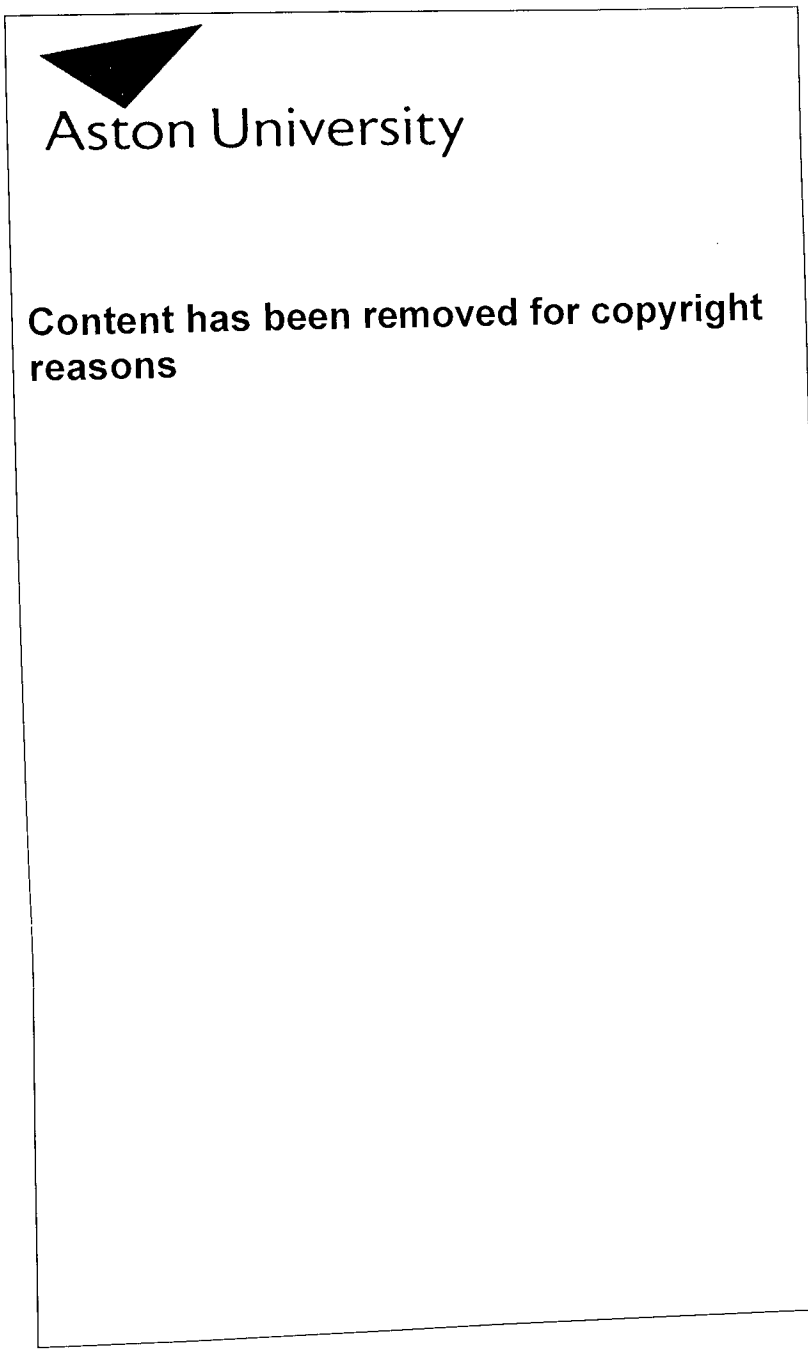
Effect of Docosahexaenoic Acid on Weight Loss

Anti-Cachectic Effect of Docosahexaenoic Acid

Beck, S.A. and Hudson, E.A.

Figure 4.15

Effect of Docosahexaenoic Acid on Tumour Volume



Anti-Tumour Effect of Docosahexaenoic Acid

Beck, S.A. and Hudson, E.A.

Figure 4.16

Anti-Tumour Effect of Gamma Linolenic Acid

Beck, S.A.

may inhibit growth of the MAC16 tumour and subsequently reduce the cachexia associated with this tumour. Therefore, since the anti-tumour and anti-cachectic effects of EPA may be independent processes with the inhibition of cachexia producing an additional reduction in tumour growth, further studies into the mechanism of action of EPA in cachectic tumour-bearing subjects will concentrate on the ability of EPA to antagonize the MAC16 lipolytic factor at the level of the adipocyte.

4.3.1.2 Investigation of the Mechanism of the Anti-Lipolytic Action of Eicosapentaenoic Acid in Cancer Cachexia

4.3.1.2.1 Investigation of the Interaction of Purified MAC16 Lipolytic Factor with the Adenylate Cyclase Signal Transduction Pathway

4.3.1.2.1.1 Introduction

The hormonal control of fat cell lipolysis occurs in two stages. Hormone molecules (first messengers) bind to cell surface receptors. The changes induced as a result of the receptor site being occupied lead to the activation of effector molecules via signal transducing proteins (G-proteins, the discovery and characterization of which is discussed in more detail in Section 1.5) and the subsequent production of second messenger molecules within the cell. These second messengers elicit the physiological response attributed to the hormone. Cyclic AMP is a key regulatory molecule in most mammalian tissues and, in the case of adipocyte lipolysis, is the molecule which effects the greatest lipid-regulating response to hormonal stimulation. Normal control of fat cell lipolysis is attributable to the action of hormones such as adrenaline, noradrenaline, ACTH, thyroid stimulating hormone and glucagon, which all stimulate lipolysis, and adenosine, insulin and prostaglandin E_1 which exert an inhibitory effect on lipolysis.

Figure 4.17

Effect of Eicosapentaenoic Acid on MAC16 Tumour-Induced Lipolytic Activity

Anti-Lipolytic Effect of Eicosapentaenoic Acid

Beck, 1989

The lipolytic factor produced by several human and animal tumours including the MAC16 tumour used in these studies has been shown to produce lipolysis in isolated adipocytes and an increase in cAMP in intact cells. Eicosapentaenoic acid, which inhibits catecholamine- and lipolytic factor-induced lipolysis (fig. 4.17), also reduces the raised cAMP levels attributed to these lipolytic substances (Tisdale and Beck, 1991). This discovery suggested the possible involvement of the stimulatory G-protein, G_s, in the mechanism of action of the MAC16 lipolytic factor and an interaction of EPA with G_i, the inhibitory G-protein of the adenylate cyclase system. The effect of purified MAC16 lipolytic factor on adenylate cyclase activity in mouse adipocyte plasma membranes was investigated in this study. The method of membrane preparation (Belsham et al, 1980) separated the plasma membrane and mitochondrial fractions by means of a self-forming percoll gradient. The purity of the membrane fractions was assessed by their enzyme activity (see Sections 3.3.1.2.1.1 to 3.3.1.2.1.4 and Belsham et al (1980) for methods and expected purity). The plasma membrane fraction, determined by 5'-nucleotidase activity, was washed three times with NaCl Buffer (Section 2.3.10) before use in the assay. A preliminary study with eicosapentaenoic acid determined whether this fatty acid modulated the activity of adenylate cyclase stimulated by the lipolytic factor.

4.3.1.2.1.2 Results

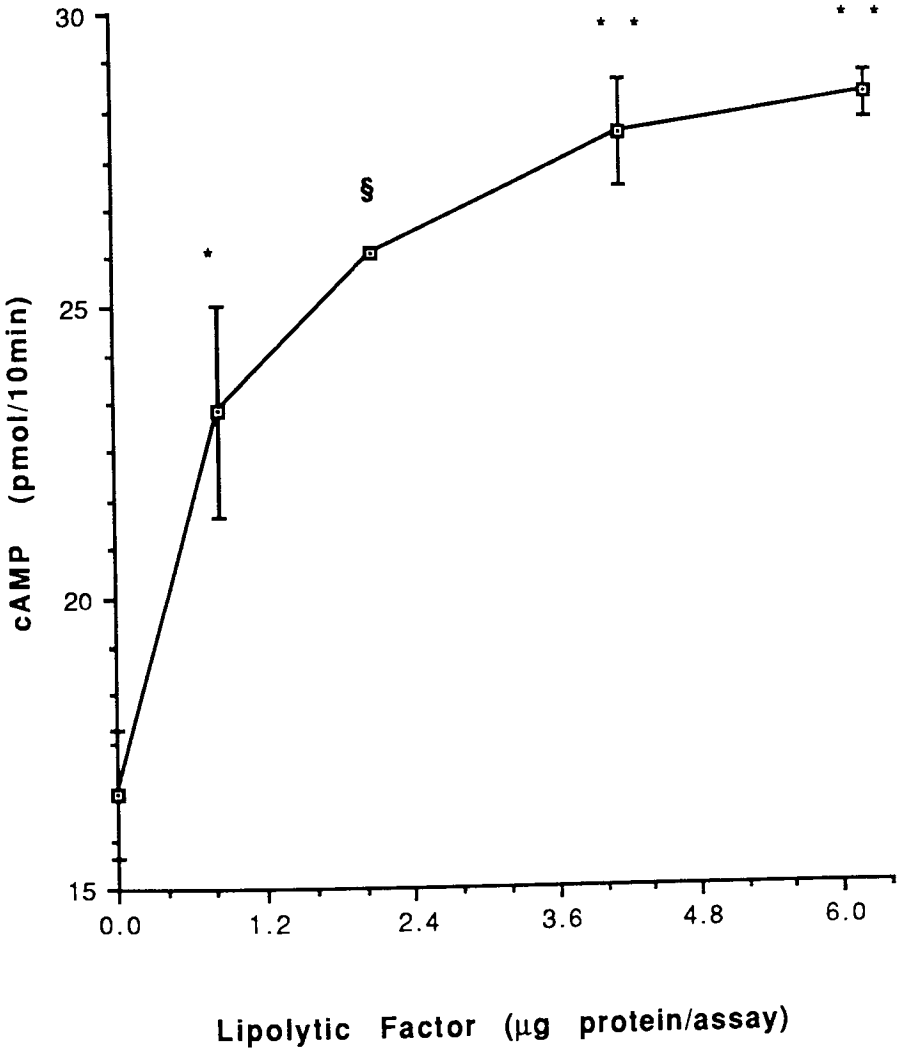
The purified MAC16 lipolytic factor stimulated adenylate cyclase activity in mouse adipocyte plasma membranes. A dose-response relationship was observed with significant stimulation by lipolytic factor in a concentration range 0.83 - 6.23μg protein (fig. 4.18). The time course for stimulation of adenylate cyclase activity (fig. 4.19) showed an increase in activity up to 10 min, after which a decrease in activity was noted. The adenylate cyclase activity had returned to basal after 120 min. A significant inhibition of MAC16 lipolytic factor - stimulated adenylate cyclase activity was produced by EPA (331μM) (fig. 4.20).

4.3.1.2.1.3 Discussion

Purified MAC16 lipolytic factor exerts a stimulatory effect on adipocyte adenylate cyclase. This effect is dose-related and increases over a time period of 10 min. The decrease in activity observed after this time point is not consistent with the result obtained by Tisdale and Beck (1991) in which an increase in cAMP levels over a period of 2 hr was observed when isolated adipocytes were incubated with a crude preparation of the MAC16 lipolytic factor. However it should be noted that the study quoted above involved the measurement of cAMP levels, as opposed to adenylate cyclase activity, and also that the study was conducted with whole adipocytes. The

Figure 4.18

Dose-response Relationship for Purified MAC16 Lipolytic Factor



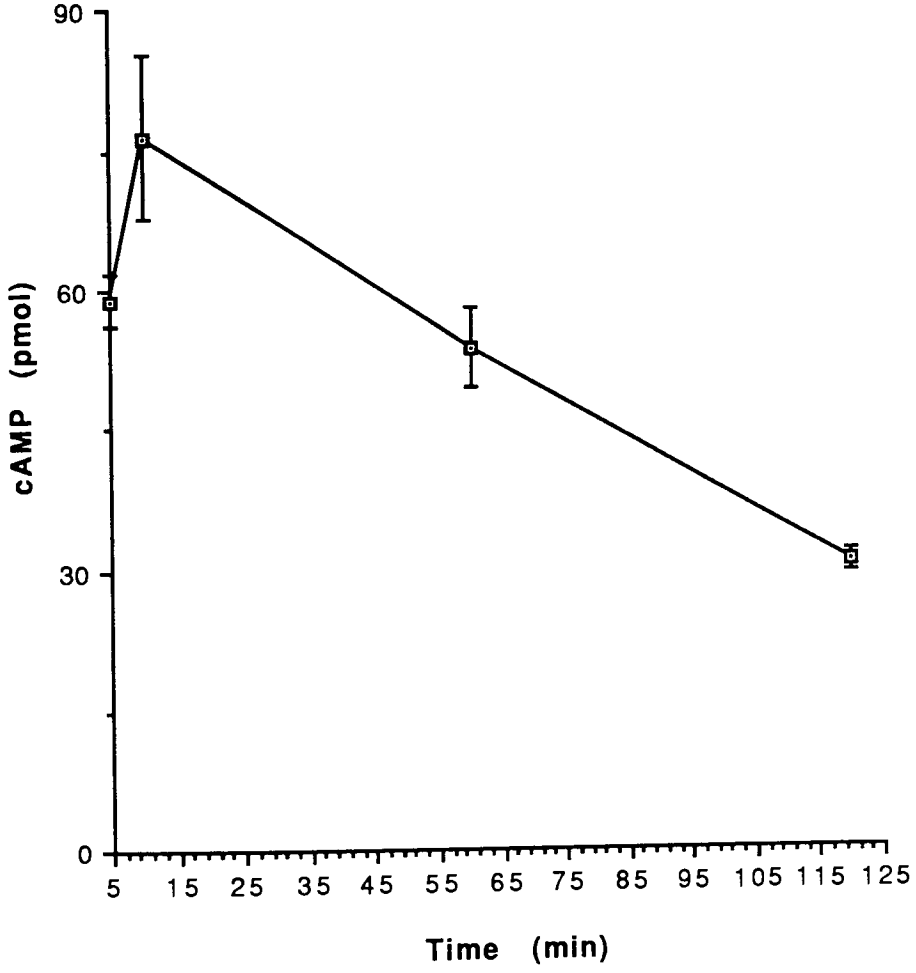
Results are expressed as mean ± SEM of 3 experiments. § = mean of 2 experiments.

Membrane concentration = 100µg/100µl

*p < 0.05, **p < 0.001 (compared with basal, STT)

Figure 4.19

Time Course for the Action of Purified MAC16 Lipolytic Factor on Adenylate Cyclase Activity



Results are expressed as mean ± SEM of 3 experiments.

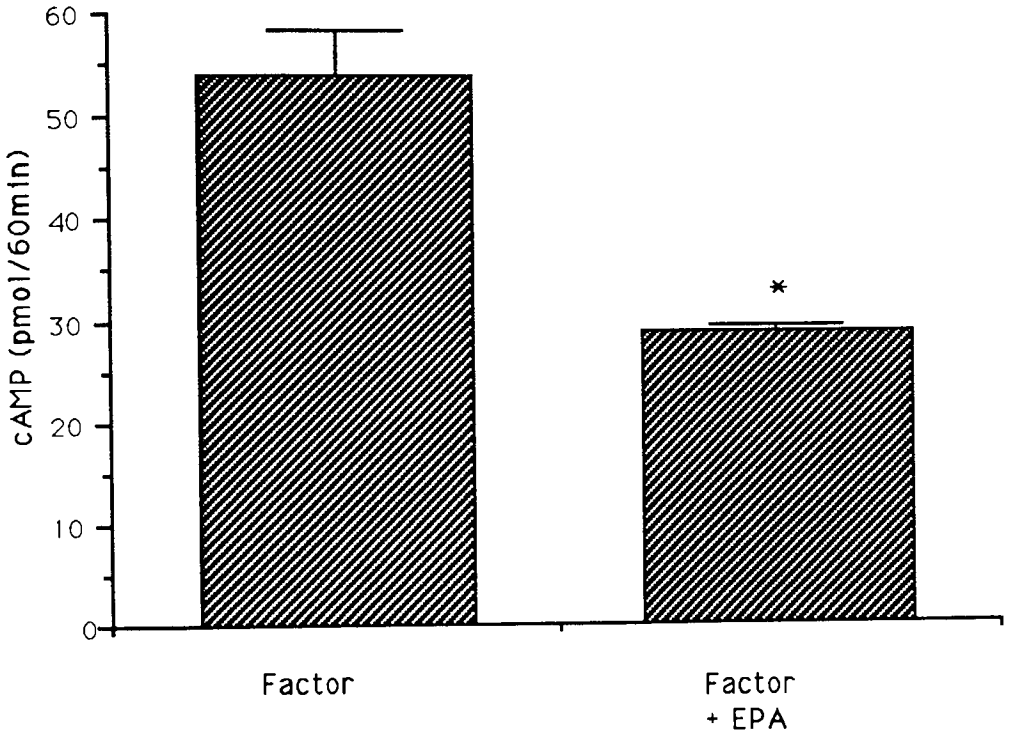
Protein content of lipolytic factor = 2.08µg/assay.

Membrane concentration = 100µg/100µl.

Basal adenylate cyclase activity + 39.1 pmol cAMP/10 min (mean of 2 experiments).

Figure 4.20

Effect of Eicosapentaenoic Acid on MAC16 Lipolytic Factor - Induced cAMP Production



Results are expressed as mean ± SEM of 3 experiments.

Concentration of EPA = 331μM.

Protein content of lipolytic factor = 2.08μg/assay.

Membrane concentration = 100μg/100μl.

Basal adenylate cyclase activity = 39.1 pmol cAMP/10 min (mean of 2 experiments).

present study utilized adipocyte plasma membranes, the preparation of which could have affected the binding of the lipolytic factor to its active site. The increase in cAMP production observed with the MAC16 lipolytic factor could explain its lipolytic effect *in vitro*, which could, in turn, determine the role of this factor in the cachectic process of MAC16 tumour-bearing animals (Beck and Tisdale, 1987). Knowledge of the mechanism of action of the lipolytic factor provides a basis for an investigation into agents which may inhibit the cachexia observed in malignant disease. Eicosapentaenoic acid, which has been shown to inhibit the cachectic effect of the MAC16 tumour *in vivo* and its lipolytic action *in vitro* (Tisdale and Beck, 1991), reduced the cAMP production due to the lipolytic factor in the present study. A phosphodiesterase inhibitor was included in the reaction mixture to eliminate the possibility of an increase in the activity of this enzyme causing a decrease in cAMP levels. This observation supports the suggestion that EPA may act via G_i, the inhibitory G-protein of the adenylate cyclase system, and provides the basis for a more detailed investigation into the mechanism of the anti-lipolytic effect of this fatty acid which will be shown in subsequent sections.

4.3.1.2.2 Investigation of the Interaction of Eicosapentaenoic Acid with G_i in the Adipocyte Plasma Membrane

4.3.1.2.2.1 Introduction

The results presented in Section 4.3.1.2.1 indicate a possible interaction of EPA with G_i, the inhibitory G-protein of the adenylate cyclase system. Such an interaction may explain the inhibitory effect of this fatty acid on lipolysis stimulated by the MAC16 lipolytic factor.

Rothenberg and Kahn (1988) showed that insulin, an anti-lipolytic hormone, interacted with a 42kDa protein in rat adipocyte and hepatocyte plasma membranes and prevented ADP-ribosylation due to pertussis toxin. This protein was identified as the α-subunit of G_i. The actions of insulin had previously been attributed to intracellular protein phosphorylation by the tyrosine kinase moiety of the insulin receptor and also to activation of cAMP phosphodiesterase (Lönnroth and Smith, 1986). Thus the study by Rothenberg and Kahn demonstrated a novel interaction between the insulin receptor and G_i which appeared to be independent of tyrosine kinase phosphorylation of the G-protein. As insulin is known for its anti-lipolytic effect, a property it shares with EPA, this method was employed to investigate an interaction between EPA and G_i in rat adipocyte plasma membranes. DHA and arachidonic acid which do not possess the anti-cachectic

action of EPA were used as controls.

One-dimensional polyacrylamide gel electrophoresis was employed in order to separate proteins of weights ~ 20 kDa to 200 kDa after ADP-ribosylation of rat adipocyte plasma membranes. This technique operates on the principle that a charged molecule will move in an electric field at a rate proportional to its charge density. The structure of the polyacrylamide gel utilized in this study arose from the polymerization of acrylamide molecules and the cross-linking of these polymers with N,N'-methylene bisacrylamide. Polymerization of the acrylamide was initiated by ammonium persulphate and accelerated using N,N,N',N'-tetramethylethylenediamine. The concentration of acrylamide determines the pore size of the gel and hence the size of proteins able to be retained and separated on the gel. Sodium dodecyl sulphate (SDS) was a continuous phase in the gel system. This compound is a detergent which dissociates lipid-protein complexes and solubilizes the proteins, thus permitting separation and analysis. Proteins were denatured before separation by heating with an excess of SDS and dithiothreitol in order to induce dissociation into the individual polypeptide chains and to impart an equal charge to each polypeptide. The large negative charge of SDS overrides the relatively small intrinsic charge of the individual polypeptides and allows separation according to size only. A discontinuous buffer system was employed in which stacking of polypeptides in order of decreasing mobility was achieved

through a large-pore stacking gel before being separated according to molecular weight on the running gel (Hames, 1990).

Autoradiography was the technique employed in order to visualize and quantify the extent of ADP-ribosylation. This technique detects radioactivity through the formation of metallic silver from silver bromide in a photographic emulsion, the energy for this reaction being provided by ionizing radiation from the sample.

4.3.1.2.2.2 Results

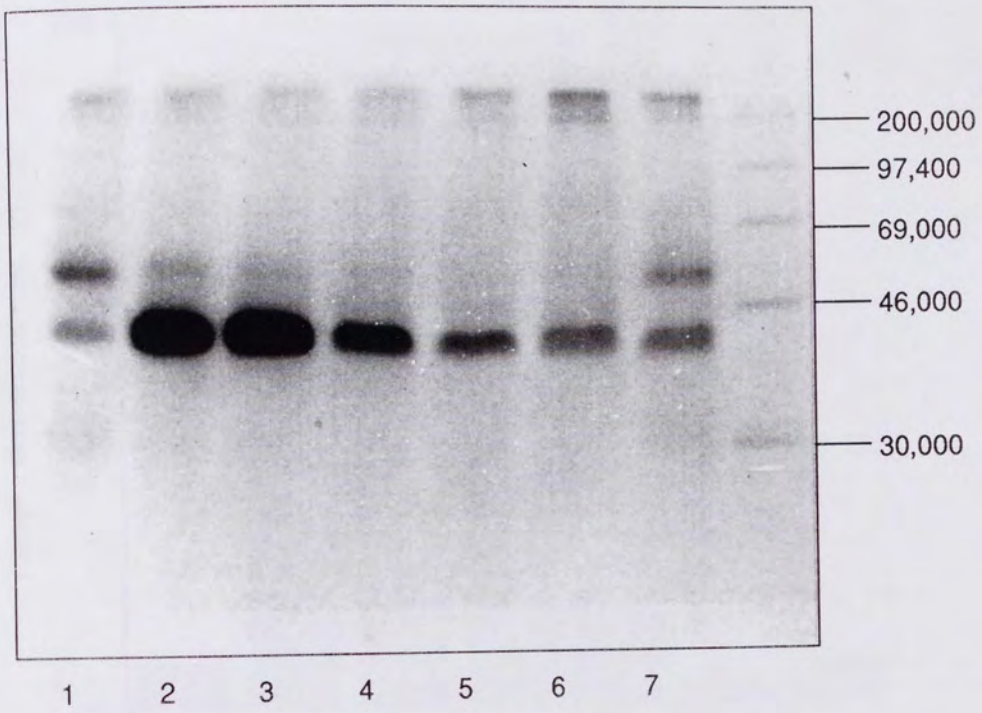
Pertussis toxin catalysed the ADP-ribosylation of a polypeptide of molecular weight approximately 40 kDa in isolated adipocyte membrane preparations. This was assumed to be the α-subunit of G_i (Murayama and Ui, 1983; Hinsch et al , 1988; Rothenberg and Kahn, 1988). Preincubation of membranes with increasing concentrations of EPA caused an initial increase in ADP-ribosylation, followed by a decrease to a level below that due to pertussis toxin alone (figs. 4.21(a) and (b)). DHA showed a similar pattern to EPA but levels of ADP-ribosylation remained considerably higher than the control (figs. 4.22(a) and (b)). Arachidonic acid also potentiated, but did not inhibit, pertussis toxin - catalysed ADP-ribosylation of G_i (figs. 4.23(a) and (b)). This potentiation increased in a dose-related manner up to a concentration of 331μM and inhibition relative to the maximum ADP-ribosylation

was only seen at a concentration of 496μM arachidonic acid. This decrease in activity was not significant when compared with the maximum value of ADP-ribosylation stimulated by arachidonic acid. ADP-ribosylation of $G_i\alpha$ was significantly higher than the control values with all fatty acids at all concentrations except EPA at 331 and 496μM. The value of the mean at 496μM EPA was approximately half that of the control but the difference was not significant. This result would therefore at first suggest that EPA did not inhibit pertussis toxin - catalysed ADP-ribosylation. However, values for EPA at a concentration of 496μM were lower than the controls in most individual preparations. The values of ADP-ribosylation with DHA or AA were not lower than the control value at any concentration in any individual preparation.

ADP-ribosylation of a polypeptide of approximate molecular weight 52 kDa, which corresponds to the molecular weight of G_s, was also unexpectedly seen (figs. 4.21(a), 4.22(a) and 4.23(a)). The pertussis toxin - catalysed ADP-ribosylation of this polypeptide was inhibited in a dose-related manner, with complete inhibition occurring at approximately 248μM EPA and DHA. AA produced inhibition to a lesser extent.

Equimolar lysine was used as a vehicle for the fatty acids. Lysine at a concentration approximately 15 times greater than that used with the fatty acids produced no alteration in the ADP-ribosylation catalysed by pertussis toxin.

Figure 4.21(a)



1 = PT (25µg/ml)

2 = PT (25µg/ml) + EPA (83µM)

3 = PT (25µg/ml) + EPA (165µM)

4 = PT (25µg/ml) + EPA (248µM)

5 = PT (25µg/ml) + EPA (331µM)

6 = PT (25µg/ml) + EPA (496µM)

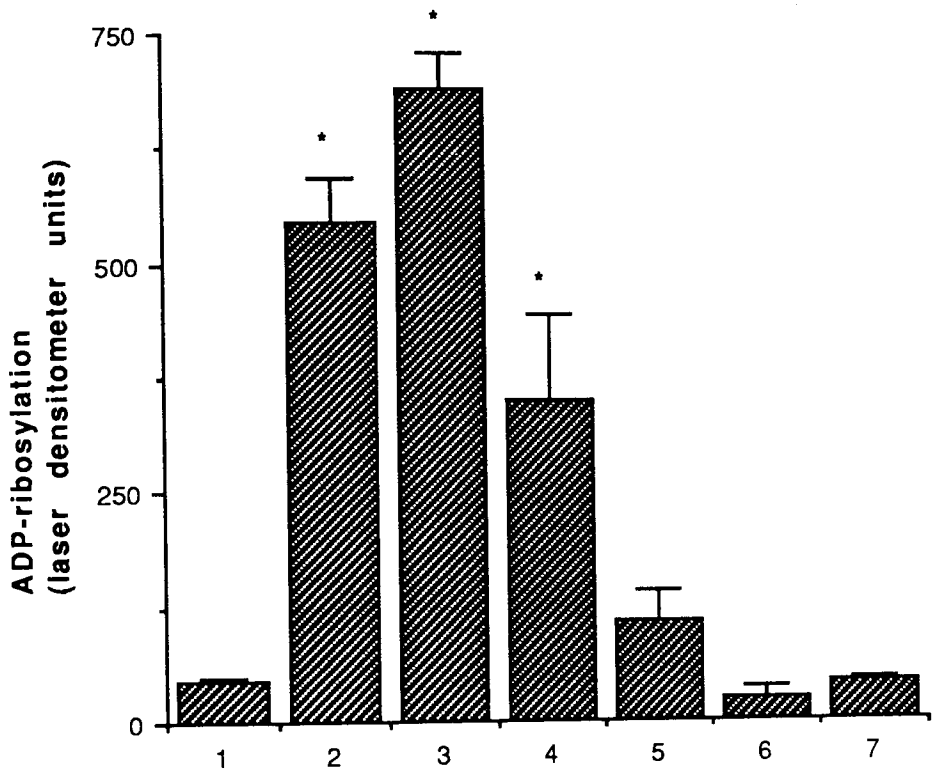
7 = PT (25µg/ml) + lysine (~10mM)

Membrane concentration = 100µg/100µl.

PT = pertussis toxin, EPA = eicosapentaenoic acid.

Figure 4.21(b)

Effect of Eicosapentaenoic Acid on ADP-Ribosylation of a 40kDa Membrane Protein by Pertussis Toxin



1 = PT (25µg/ml)

2 = PT (25µg/ml) + EPA (83µM)

3 = PT (25µg/ml) + EPA (165µM)

4 = PT (25µg/ml) + EPA (248µM)

5 = PT (25µg/ml) + EPA (331µM)

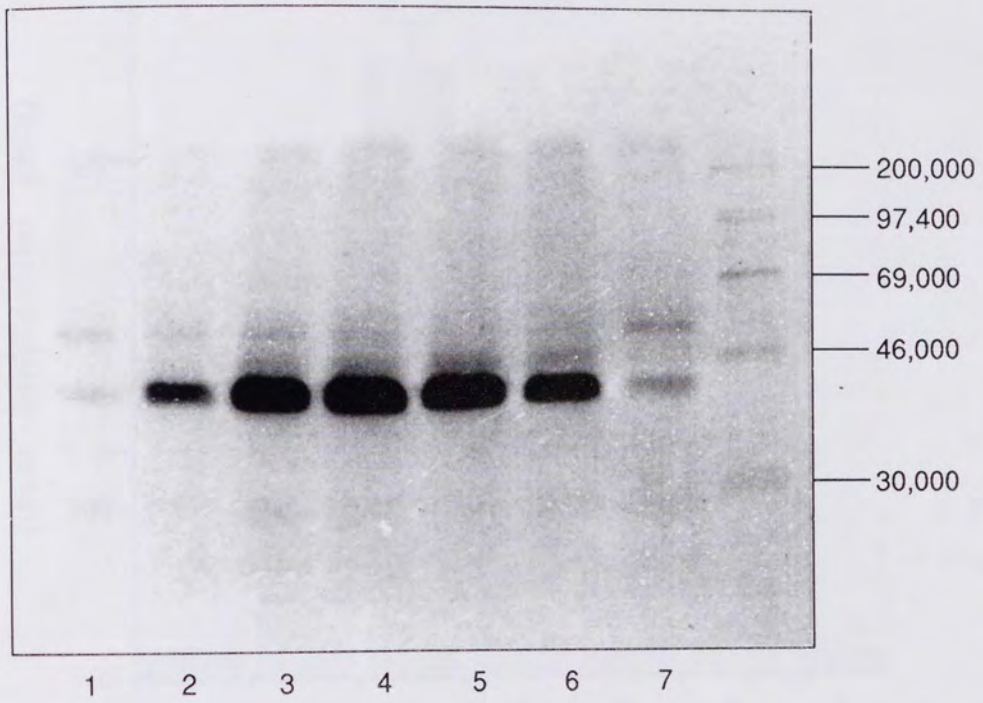
6 = PT (25µg/ml) + EPA (496µM)

7 = PT (25µg/ml) + lysine (~10mM)

Results are expressed as mean ± SEM for 3 experiments.

*p < 0.001 (compared with PT controls, STT).

Figure 4.22(a)



1 = PT (25µg/ml)

2 = PT (25µg/ml) + DHA (83µM)

3 = PT (25µg/ml) + DHA (165µM)

4 = PT (25µg/ml) + DHA (248µM)

5 = PT (25µg/ml) + DHA (331µM)

6 = PT (25µg/ml) + DHA (496µM)

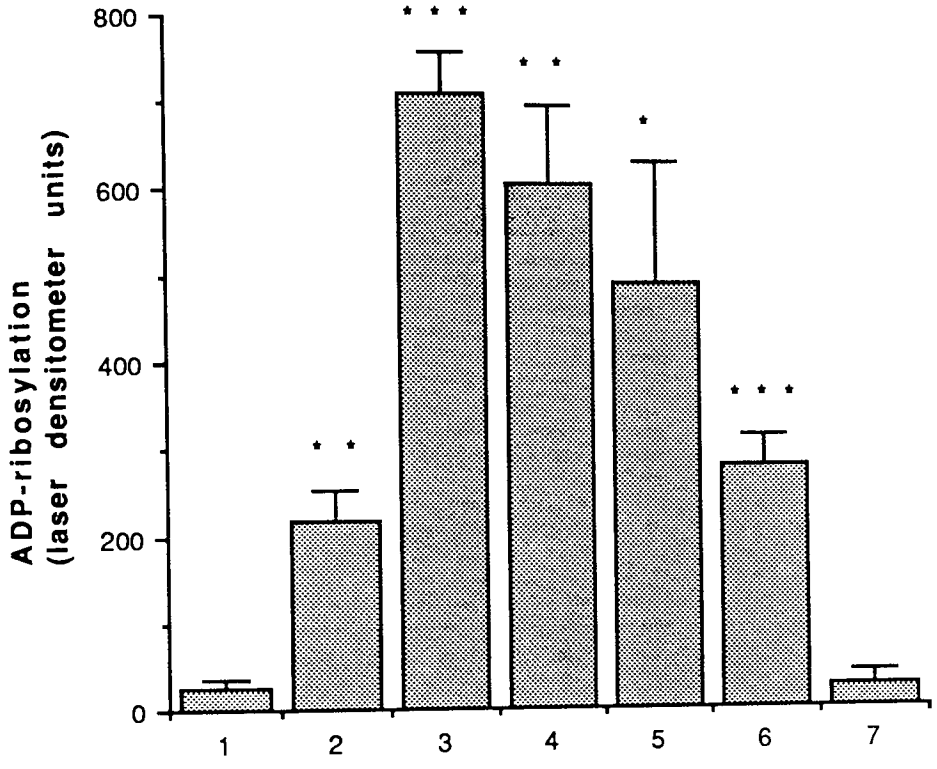
7 = PT (25µg/ml) + lysine (~10mM)

Membrane concentration = 100µg/100µl.

PT = pertussis toxin, DHA = docosahexaenoic acid.

Figure 4.22(b)

Effect of Docosahexaenoic Acid on ADP-Ribosylation of a 40kDa Membrane Protein by Pertussis Toxin



1 = PT (25µg/ml)

2 = PT (25µg/ml) + DHA (83µM)

3 = PT (25µg/ml) + DHA (165µM)

4 = PT (25µg/ml) + DHA (248µM)

5 = PT (25µg/ml) + DHA (331µM)

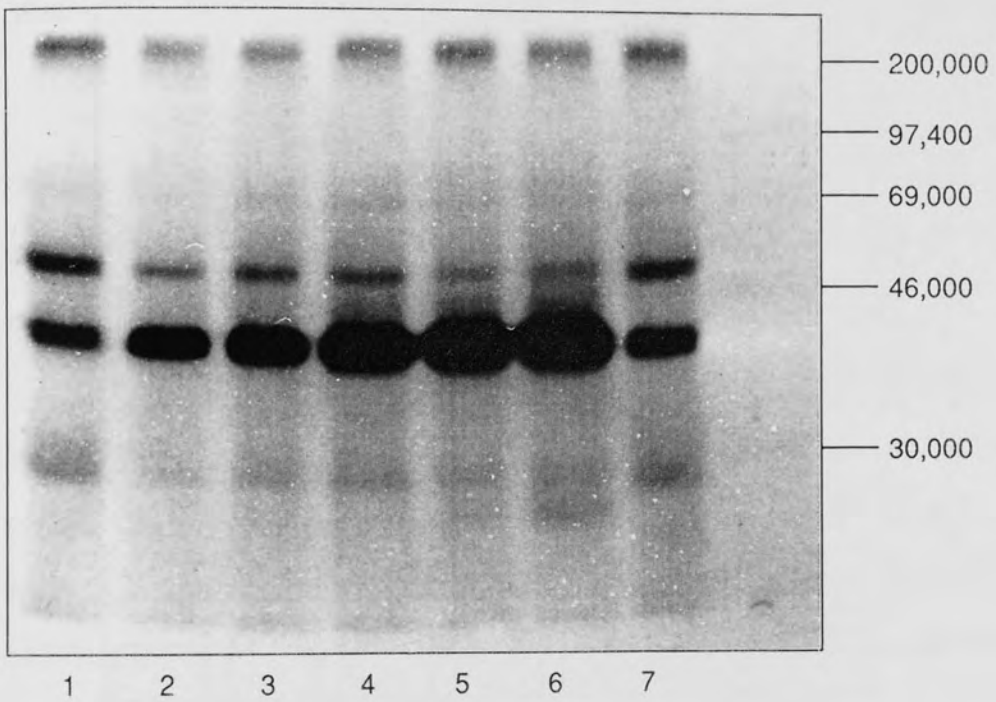
6 = PT (25µg/ml) + DHA (496µM)

7 = PT (25µg/ml) + lysine (~10mM)

Results are expressed as mean ± SEM of 3 experiments.

*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ (compared with PT controls, STT).

Figure 4.23(a)



1 = PT (25µg/ml)

2 = PT (25µg/ml) + AA (83µM)

3 = PT (25µg/ml) + AA (165µM)

4 = PT (25µg/ml) + AA (248µM)

5 = PT (25µg/ml) + AA (331µM)

6 = PT (25µg/ml) + AA (496µM)

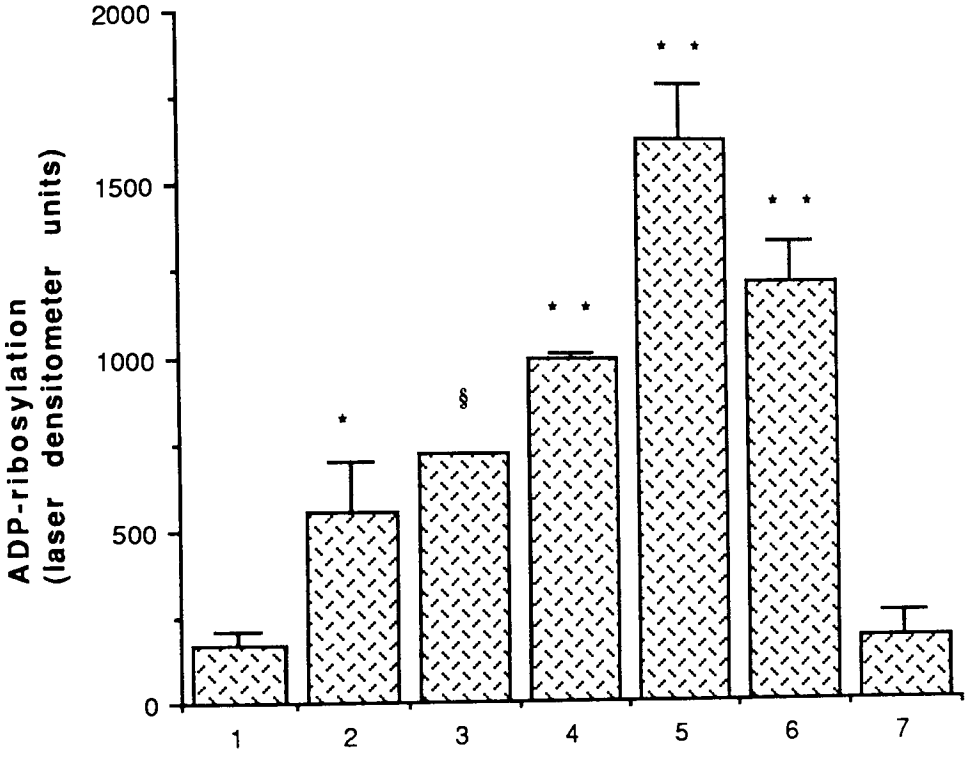
7 = PT (25µg/ml) + lysine (~10mM)

Membrane concentration = 100µg/100µl.

PT = pertussis toxin, AA = arachidonic acid.

Figure 4.23(b)

Effect of Arachidonic Acid on ADP-Ribosylation of a 40kDa Membrane Protein by Pertussis Toxin



1 = PT (25µg/ml)

2 = PT (25µg/ml) + AA (83µM)

3 = PT (25µg/ml) + AA (165µM)

4 = PT (25µg/ml) + AA (248µM)

5 = PT (25µg/ml) + AA (331µM)

6 = PT (25µg/ml) + AA (496µM)

7 = PT (25µg/ml) + lysine (~10mM)

Results are expressed as mean ± SEM of 3 experiments. *p < 0.05, **p < 0.001 (compared with PT controls, STT), § = mean of 2 experiments.

4.3.1.2.2.3 Discussion

The increase in pertussis-toxin catalysed ADP-ribosylation of the 40 kDa polypeptide was an unexpected observation and the decrease in this potentiation seen at higher concentrations was even more difficult to explain. Pertussis toxin is only able to ADP-ribosylate the inactive form of $G_{i\alpha}$ where the subunits are associated, GDP is bound and the associated receptor site is unoccupied. This may be due, in part, to the proximity of the pertussis toxin active site to the region of $G_i\alpha$ which interacts with receptors (Sullivan et al, 1987), which would also explain why G-proteins which are substrates for pertussis toxin are functionally uncoupled from their receptors after treatment with this toxin. Several studies have shown decreased binding of inhibitory agonists after treatment with pertussis toxin to support this theory (Kurose et al, 1983; Hsia et al, 1984; Boyer et al, 1984). However a study by Katada et al (1986) showed that ADP-ribosylation of purified α-subunits which were sensitive to pertussis toxin was not possible in the absence of $\beta\gamma$ subunits and was inhibited by the presence of Mg^{2+} (which promotes dissociation of the $\alpha\beta\gamma$ trimer), thus suggesting that ADP-ribosylation by pertussis toxin may be inhibited in the absence of a receptor - G-protein interaction. The results of this experiment therefore suggest that in low concentrations the polyunsaturated fatty acids, EPA, DHA and AA, do not interact with a receptor which is coupled to G_i and that $G_i\alpha$ is

in the inactive form. However, an interaction with $G_i\alpha$ is apparent and it appears that this interaction increases the ability of pertussis toxin to catalyse ADP-ribosylation of $G_i\alpha$, possibly by inducing a conformational change in the polypeptide which exposes the active site for pertussis toxin. The significance of this interaction is not fully understood at this stage since an alteration in the region of the α-subunit which is susceptible to ADP-ribosylation by pertussis toxin would be expected to cause uncoupling of the receptors associated with G_i and remove the inhibitory effect of this G-protein on adenylate cyclase. An increase in lipolysis at low concentrations of EPA would therefore be expected, which was not the case (Beck, 1989). At higher concentrations, all fatty acids showed inhibition of ADP-ribosylation relative to the maximum value obtained. Arachidonic acid potentiated ADP-ribosylation up to a concentration of 331μM and began to "inhibit" at 496μM. DHA and EPA showed identical patterns of potentiation and relative inhibition with maximum ADP-ribosylation occurring at a concentration of 165μM. However, only EPA produced an inhibition of the ADP-ribosylation due to pertussis toxin alone (at 496μM). The value of ADP-ribosylation at this concentration of DHA was still considerably higher than the control value. An explanation for this observation may be that increasing concentrations of fatty acid cause a progressive alteration of the conformation of $G_i\alpha$. Assuming the deduction that the observed increase in ADP-ribosylation was due to exposure of the pertussis toxin

active site to be correct, it appears that increasing the concentration of EPA, DHA or AA in the membrane preparation further alters the conformation of $G_i\alpha$ and this new conformation is relatively unfavourable to binding of the ADP-ribose unit transferred from NAD by the toxin.

DHA and AA were included in the study since experiments investigating the anti-lipolytic properties of eicosapentaenoic acid, docosahexaenoic acid methyl ester and arachidonic acid in vitro showed that only EPA exerted an inhibitory effect on lipolysis produced by a range of agonists (Tisdale and Beck, 1991). The fact that all three fatty acids behaved in a similar manner concerning the effect on pertussis toxin - catalysed ADP-ribosylation was therefore surprising. The only difference appeared to be in the pattern and extent of ADP-ribosylation seen with these fatty acids. DHA and AA promoted favourable conditions for the action of pertussis toxin in the concentration range studied. EPA was the exception in that it inhibited ADP-ribosylation of $G_i\alpha$ at the highest concentration studied. This inhibition suggested that subunit dissociation may occur at this concentration.

It should be noted that although the inhibitory component of adenylate cyclase has been referred to as G_i throughout this study, three distinct forms of this protein, $G_{i-1,2 \text{ and } 3}$, have been identified (Mitchell et al, 1989). It was not clear until recently which of these (if not all three) was responsible for inhibition of adenylate cyclase. However, Simonds et al (1989) and McKenzie and Milligan (1990) have

suggested that possibly only G_{i-2} is involved in the adenylate cyclase system.

The appearance of a second substrate for pertussis toxin - catalysed ADP-ribosylation of approximate molecular weight 52 kDa was totally unexpected. The molecular weight of this polypeptide suggested that it may be G_s, the stimulatory G-protein in the adenylate cyclase system. Several reports have been made in which ADP-ribosylation of G_i by cholera toxin occurred in the absence of guanine nucleotides (Owens et al, 1985; Ui and Katada, 1990). It was also noted that the polypeptides which were ADP-ribosylated under these conditions were also substrates for pertussis toxin and that the functional consequences of cholera toxin - catalysed ADP-ribosylation of G_i were the same as those produced by pertussis toxin - treatment of this polypeptide (Owens et al, 1985). However, despite this observation, pertussis and cholera toxins appear to have different sites of action on $G_i\alpha$ (Ui and Katada, 1990). This phenomenon was attributed to the fact that the site of action of cholera toxin, a highly conserved arginine residue in the α-subunit of G-proteins, is in close proximity to the guanine nucleotide binding site in some G-proteins. These G-proteins are therefore not substrates for cholera toxin in the presence of guanine nucleotides, but may become so by removal of the nucleotides (Milligan, 1988). The explanation of the exposed site for cholera toxin - catalysed ADP-ribosylation in the absence of guanine nucleotides, and the implied ability of all G-proteins to undergo ADP-

ribosylation catalysed by this toxin, does not apply to this case in which G_s was apparently ADP-ribosylated by pertussis toxin. As stated previously, the primary structure around equivalent arginine residues ADP-ribosylated by cholera toxin is highly conserved in all known G-proteins (Milligan, 1988). This is not the case with the site of action of pertussis toxin, which is a cysteine residue close to the carboxy-terminus of the α-subunit. No reports have so far been made of pertussis toxin - catalysed ADP-ribosylation of G_s. Milligan (1987) reported ADP-ribosylation by cholera toxin of a 40 kDa pertussis toxin substrate which was not the α-subunit of G_i, G_o or transducin. The phenomenon observed in this study may be explained by the apparent similarity between all known G-proteins. Transducin and G_i are known to possess a very high degree of homology, and G_s appears to be similar to G_i (Manning and Gilman, 1983). Since transducin is able to undergo ADP-ribosylation by pertussis and cholera toxins, it appears that very subtle changes in the α-subunit structure and amino acid composition are responsible for the susceptibility of these polypeptides to bacterial toxins. Therefore it is possible that a site for pertussis toxin - catalysed ADP-ribosylation exists on G_s and is accessible under certain conditions. Why this should occur in this experiment and not in the experiment described by Rothenberg and Kahn (1988) which was very similar, is not clear. One explanation may be that the specific activity of [α-^{32}P]NAD was much higher in this experiment and perhaps this is required in order to see the effect of

pertussis toxin on G_s. The effect of the fatty acids on this polypeptide was interesting. Inhibition of ADP-ribosylation occurred with EPA, DHA and AA. Assuming it to be G_s, this result indicates an additional interaction with the adenylate cyclase system which may have functional consequences.

4.3.1.2.3 Investigation of the Interaction of Eicosapentaenoic Acid with Adipocyte Adenylate Cyclase and its Link to Adipocyte Lipolysis

4.3.1.2.3.1 Introduction

The results presented in Section 4.3.1.2.2 suggest a non-selective interaction of polyunsaturated fatty acids with G_i, the inhibitory G-protein coupled to adenylate cyclase. Of the three fatty acids studied, only EPA produces an anti-cachectic effect *in vivo* (Tisdale and Beck, 1991). A study by Gold (1978) revealed that clofibrate, a drug which is used clinically to lower serum triglycerides, exhibited an anti-tumour effect against the Walker 256 carcinoma. This drug, when fed to rats, has been shown to significantly inhibit adenylate cyclase activity in several tissue types including adipose tissue (Greene *et al*, 1970). It is therefore possible that clofibrate may be inhibiting tumour growth by reducing the supply of glycerol from adipose tissue for conversion to glucose, this being proposed as a significant factor in advancing cancer (Gold, 1978). Alternatively, the decrease in adipocyte adenylate cyclase activity may reduce the release of tumour-promoting fatty acids from adipose stores. The object of the present investigation is therefore to determine the relationship between the anti-cachectic effect of EPA and its interaction with adipocyte adenylate cyclase, using DHA and AA as controls.

Alterations in adenylate cyclase activity and lipolysis due to an alteration in the plasma membrane lipid profile have been noted in the past. Laustolia et al (1986) reported that cardiac muscle from rats fed a cod liver oil - supplemented diet showed a reduced level of cAMP under basal and noradrenaline-stimulated conditions but suggested that the responsiveness of adenylate cyclase to catecholamines in cod liver oil - treated animals was higher despite the lower basal cAMP levels. These results were very similar to those obtained by Parrish et al (1991) who noted changes in adenylate cyclase activity in rats fed a diet supplemented with fish oil. A reduction in basal lipolysis was noted with an elevation of isoprenaline-stimulated lipolysis in adipocytes isolated from the perirenal and epididymal fat pads of fish oil - treated rats. These changes were attributed to observed alterations in the lipid profile of the adipocyte plasma membrane. Pasquier et al (1988) reported that adipocytes isolated from ovariectomized rats treated with oestrogen exhibited increased basal and stimulated lipolysis which corresponded with increased adenylate cyclase activity. The effect on adenylate cyclase in this case was thought to be due to increased catalytic activity of this enzyme. It was noted that the observed effect may have been due to oestrogen-induced hypercholesterolaemia which could have altered the composition of the adipocyte plasma membrane in the area close to the adenylate cyclase catalytic site and increased the activity of the enzyme. This suggestion is supported by the

observation by Sinensky et al (1979) that plasma membrane adenylate cyclase activity increased with increasing membrane cholesterol content. In addition to these studies, Orly and Schramm (1975) and Engelhard et al (1976) noted an increase in the activity of adenylate cyclase in turkey erythrocyte and mouse fibroblast plasma membranes respectively; this being attributed, at least in part, to the incorporation of unsaturated fatty acids into the membranes. The suggestion that lipids may interact with G_i was made by Proll et al (1985) who reported that monooleylphosphatidate inhibited adenylate cyclase activity in fibroblasts in a GTP-dependent manner, this inhibition being abolished by pertussis toxin treatment.

In the present study, the effects of EPA, DHA and AA on adenylate cyclase were compared with their action on lipolysis in vitro and anti-cachectic effect in vivo. Supplies of purified lipolytic factor were limited and costly for an experiment of this size. Therefore, since EPA has been shown to inhibit a wide range of lipolytic substances including catecholamines and the MAC16 lipolytic factor (Tisdale and Beck, 1991), isoprenaline was used as a model for lipolysis and associated cAMP increase. The extent of incorporation of EPA into rat adipocyte plasma membranes was also measured. All adenylate cyclase assays were performed on rat adipocyte plasma membrane preparations and all lipolysis assays were performed using isolated mouse adipocytes unless otherwise stated in the results.

4.3.1.2.3.2 Results

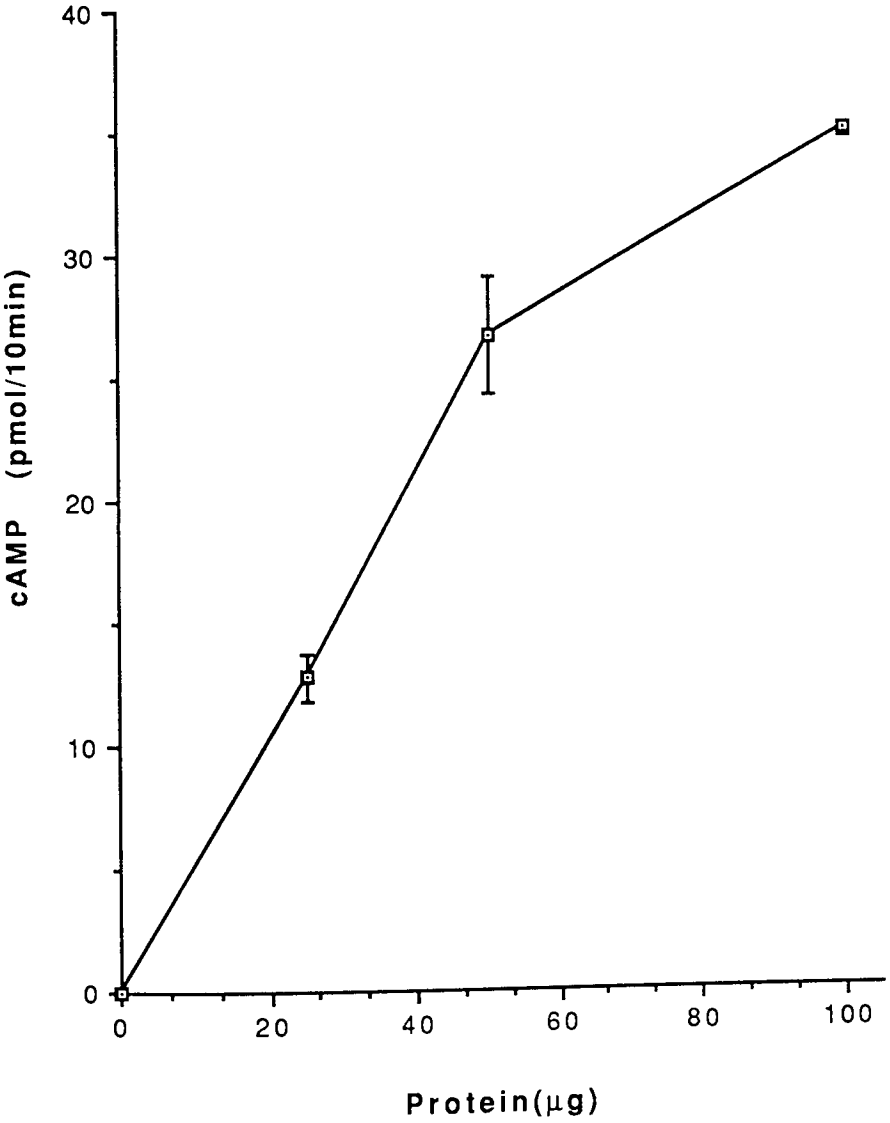
Isoprenaline produced a dose-related increase in adenylate cyclase activity up to 100µg membrane protein (fig. 4.24).

When the concentration of adipocyte plasma membrane protein in the assay was 100µg/100µl, EPA, DHA, and AA caused a significant increase in isoprenaline-stimulated adenylate cyclase activity (figs. 4.25(a), (b) and (c)). With 75µg membrane protein, all three fatty acids produced an increase in adenylate cyclase activity in low concentrations, with this activity decreasing at higher concentrations (figs. 4.26(a), (b) and (c)). Arachidonic acid differed from EPA and DHA in that the stimulation produced by this fatty acid was more highly significant and also because, unlike the other two fatty acids, it did not produce a significant decrease in adenylate cyclase activity stimulated by isoprenaline at a concentration of 496µM. With 50µg plasma membranes, a distinct dose-related inhibition of isoprenaline-stimulated adenylate cyclase activity was observed with all three fatty acids (figs. 4.27(a), (b) and (c)). This inhibition was significant at a concentration of 165µM EPA and DHA and at 248µM AA. With 25µg (figs. 4.28(a), (b) and (c)) and 10µg plasma membranes (figs. 4.29(a), (b) and (c)), inhibition of stimulated adenylate cyclase activity was still observed although it was not dose-related at this concentration of membrane protein.

The time course for stimulation (with 100µg membrane

Figure 4.24

Effect of Increasing Plasma Membrane Protein Concentration on cAMP Production



Results are expressed as mean ± SEM of 3 experiments.

Isoprenaline concentration = 1µM.

Basal adenylate cyclase activity = 21.5 ± 0.9 pmol cAMP/100µg protein/10 min (mean ± SEM of 3 experiments).

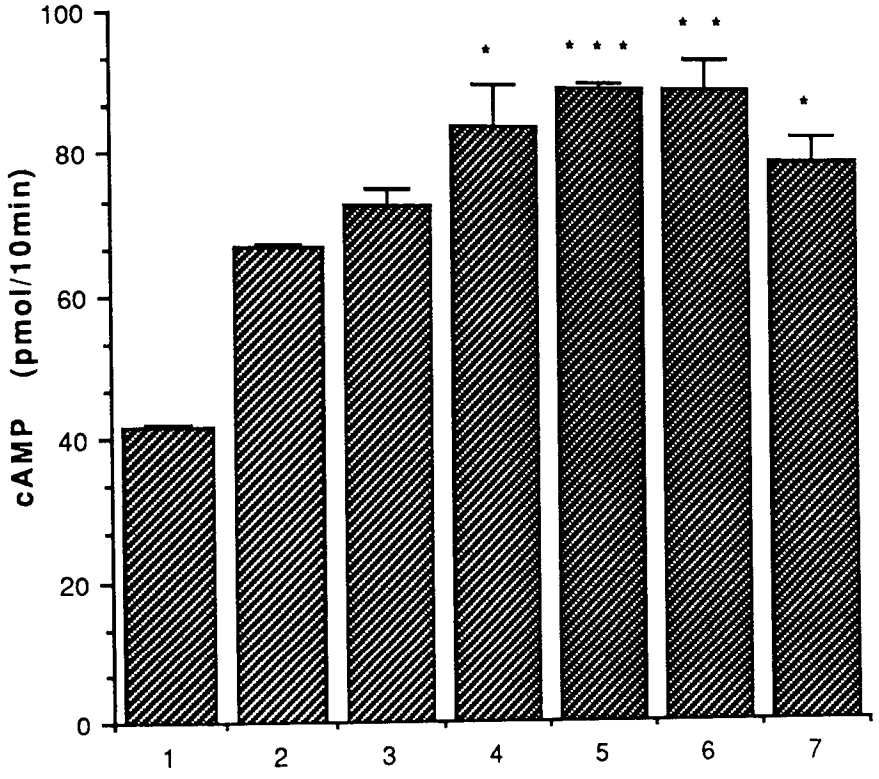
protein) and inhibition (with 50μg membrane protein) of isoprenaline-stimulated adenylate cyclase activity is shown in figs. 4.30 and 4.31 respectively. Enhancement of isoprenaline-stimulated adenylate cyclase activity remained fairly constant (110 - 120% of adenylate cyclase activity) from 0 - 10 min and increased to approximately 155% at the 60 min time point. Inhibition of isoprenaline-stimulated cAMP production by EPA was fairly constant (70 - 80% of adenylate cyclase activity) from 0 - 20 min and decreased to approximately 50% at the 60 min time point.

Stimulation of adenylate cyclase activity by EPA decreased from approximately 235% under basal conditions to approximately 140% at 1 - 2μM isoprenaline (fig. 4.32). Inhibition of adenylate cyclase activity was relatively constant (approximately 30 - 40%) with concentrations of isoprenaline ranging from 0 - 0.4μM, with the degree of inhibition increasing to approximately 55% at 1μM isoprenaline (fig. 4.33).

The results presented in figs. 4.34(a) and (b) show the effect of increasing GTP concentration on the inhibition of cAMP production by 1μM isoprenaline in the presence or absence of either EPA or DHA. Whereas an increase in isoprenaline-stimulated activity was observed corresponding to an increase in GTP concentration, the level of stimulation with fatty acid - treated plasma membranes remained fairly constant. An increasingly significant difference between the two sets of values was observed with increasing GTP

Figure 4.25(a)

Effect of Eicosapentaenoic Acid on cAMP Production with 100μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + EPA (83μM)

4 = isoprenaline (1μM) + EPA (165μM)

5 = isoprenaline (1μM) + EPA (248μM)

6 = isoprenaline (1μM) + EPA (331μM)

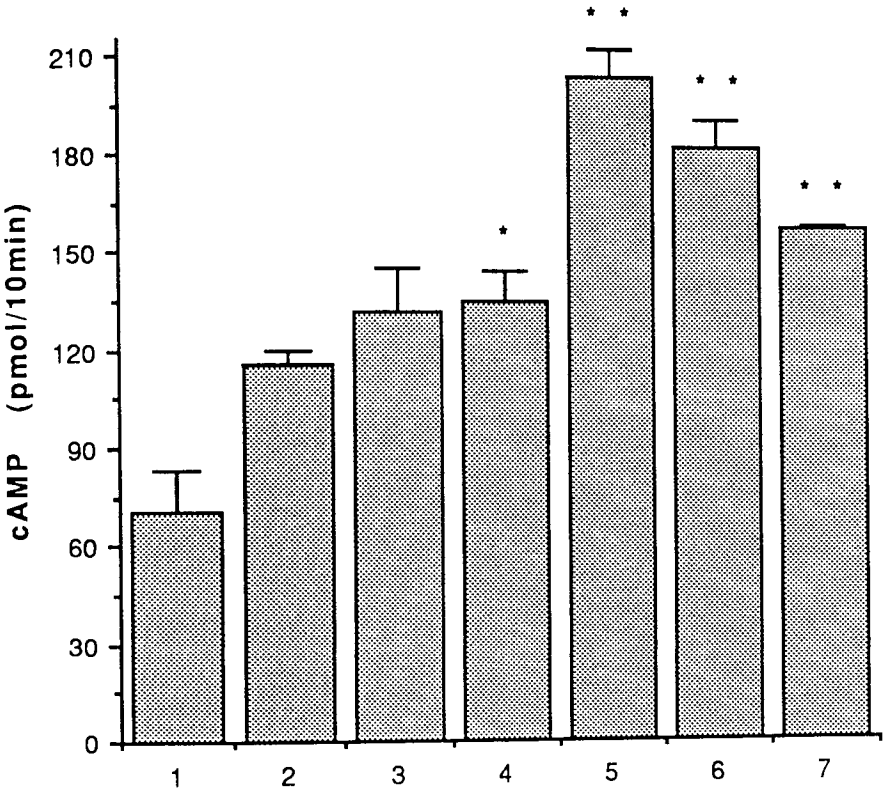
7 = isoprenaline (1μM) + EPA (496μM)

Results are expressed as mean ± SEM of 3 experiments.

*p < 0.05, **p < 0.005, ***p < 0.001 (compared with iso, STT).

Figure 4.25(b)

Effect of Docosahexaenoic Acid on cAMP Production with 100μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + DHA (83μM)

4 = isoprenaline (1μM) + DHA (165μM)

5 = isoprenaline (1μM) + DHA (248μM)

6 = isoprenaline (1μM) + DHA (331μM)

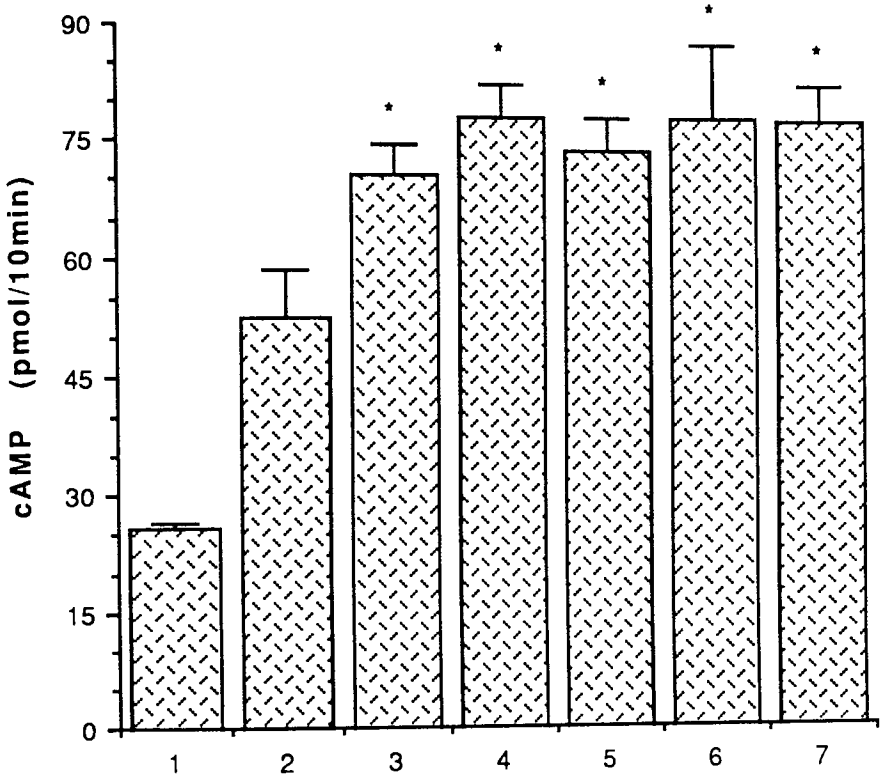
7 = isoprenaline (1μM) + DHA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments.

*p < 0.05, **p < 0.001 (compared with iso, STT).

Figure 4.25(c)

Effect of Arachidonic Acid on cAMP Production with 100µg Plasma Membranes



1 = basal

2 = isoprenaline (1µM)

3 = isoprenaline (1µM) + AA (83µM)

4 = isoprenaline (1µM) + AA (165µM)

5 = isoprenaline (1µM) + AA (248µM)

6 = isoprenaline (1µM) + AA (331µM)

7 = isoprenaline (1µM) + AA (496µM)

Results are expressed as mean ± SEM of 3 experiments.

*$p < 0.05$ (compared with iso, STT).

concentration.

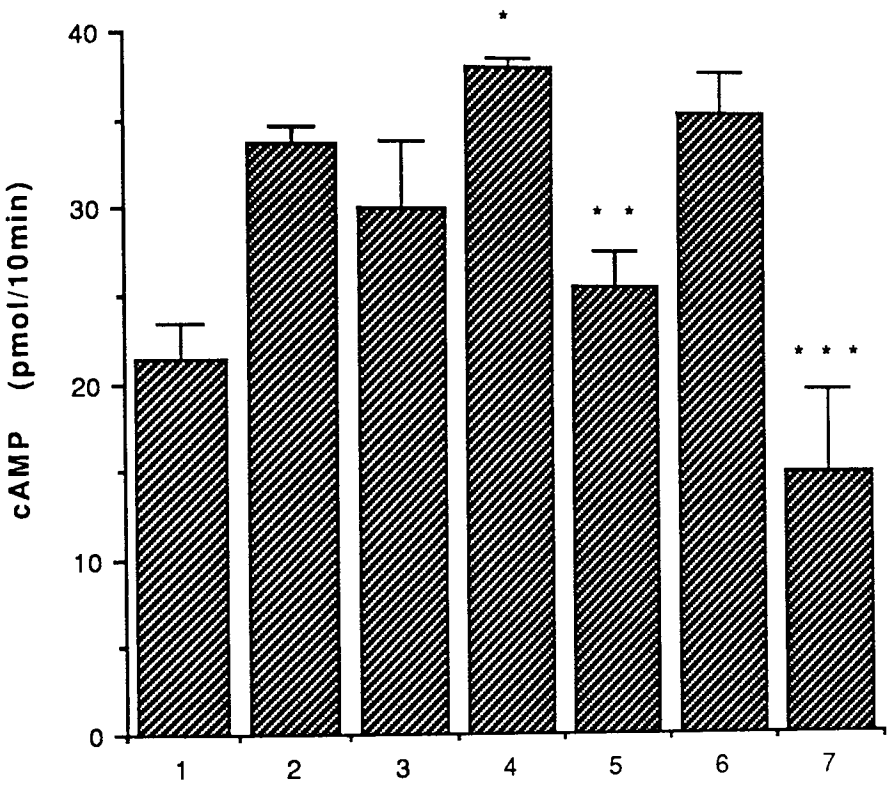
The results presented in figs. 4.35(a), (b) and (c) show the effect of EPA, DHA and AA on isoprenaline-stimulated lipolytic activity in rat adipocytes. All three fatty acids inhibited lipolytic activity. The inhibition produced by EPA and DHA was dose - related and highly significant at 496μM fatty acid ($p < 0.005$), whereas the inhibition due to AA showed no dose - dependency in the same concentration range and the inhibition at 496μM was less significant ($p < 0.05$).

The results presented in figs. 4.36(a) and (b) show the concentration-dependent incorporation of EPA into rat adipocyte plasma membranes. Approximately 50% of the EPA added to the incubation mixture was incorporated into the membranes and the remaining EPA was externally associated with the membranes as determined by analysis of the membrane washes. No EPA was detected in the incubation medium after 15 min at 37°C.

The results presented in fig. 4.37 show the effect of pertussis toxin treatment on the inhibition of adenylate cyclase produced by EPA, DHA and AA. Although the inhibition was not completely abolished by pertussis toxin treatment for any fatty acid (it was reversed to the basal level of cAMP production), and the differences between values for pertussis toxin - treated and untreated membranes were of similar significance to values for basal and isoprenaline-stimulated cAMP production, the percentage increase in cAMP production due to pertussis toxin treatment was higher for the fatty acid

Figure 4.26(a)

Effect of Eicosapentaenoic Acid on cAMP Production with 75μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + EPA (83μM)

4 = isoprenaline (1μM) + EPA (165μM)

5 = isoprenaline (1μM) + EPA (248μM)

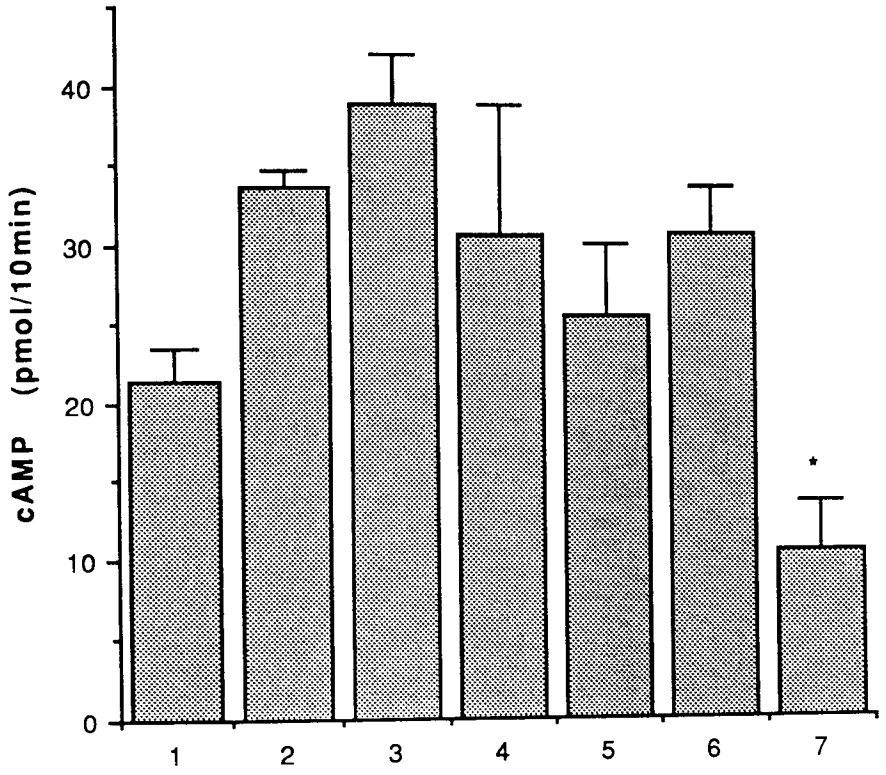
6 = isoprenaline (1μM) + EPA (331μM)

7 = isoprenaline (1μM) + EPA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments. *p < 0.05, **p < 0.01, ***p < 0.005 (compared with iso, STT).

Figure 4.26(b)

Effect of Docosahexaenoic Acid on cAMP Production with 75μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + DHA (83μM)

4 = isoprenaline (1μM) + DHA (165μM)

5 = isoprenaline (1μM) + DHA (248μM)

6 = isoprenaline (1μM) + DHA (331μM)

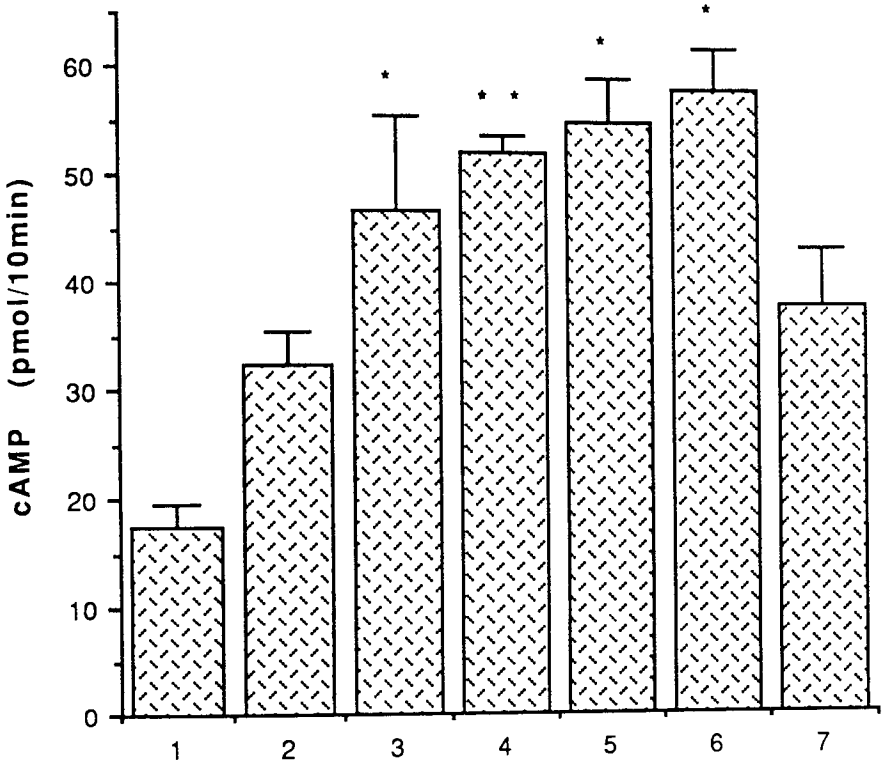
7 = isoprenaline (1μM) + DHA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments.

*$p < 0.001$ (compared with iso, STT).

Figure 4.26(c)

Effect of Arachidonic Acid on cAMP Production with 75μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + AA (83μM)

4 = isoprenaline (1μM) + AA (165μM)

5 = isoprenaline (1μM) + AA (248μM)

6 = isoprenaline (1μM) + AA (331μM)

7 = isoprenaline (1μM) + AA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments.

*p < 0.005, **p < 0.001 (compared with iso, STT).

- treated samples (ie. basal and isoprenaline levels were increased by approximately 185 - 200%; levels of cAMP production for fatty acid - treated membranes were increased by approximately 300 - 500% by pertussis toxin treatment).

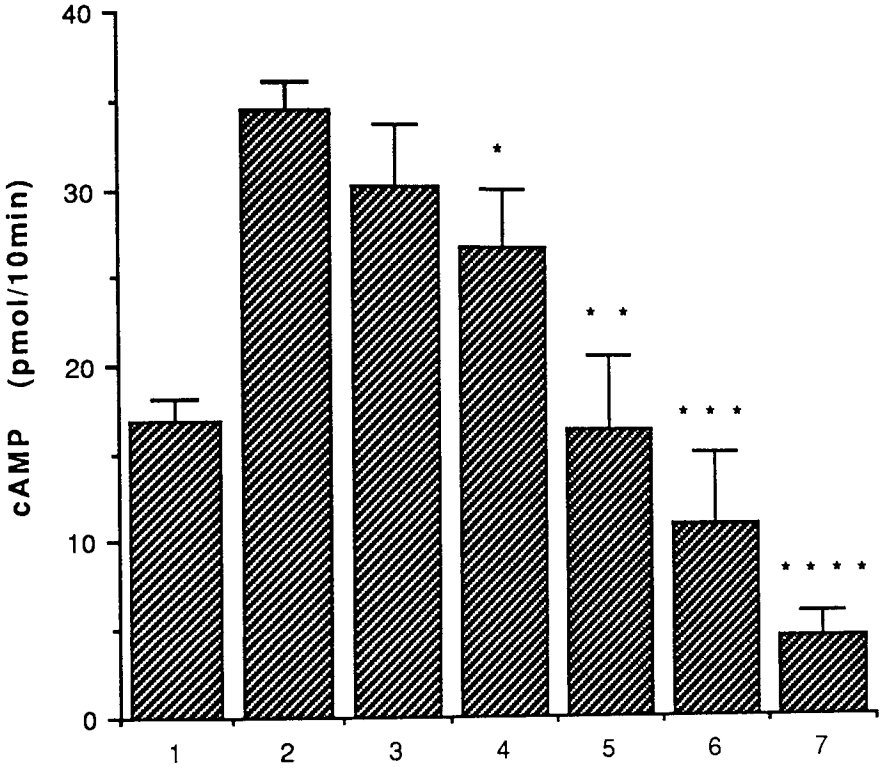
Pertussis toxin treatment did not abolish the inhibition of isoprenaline-stimulated lipolysis in mouse adipocytes (increase in lipolysis was 155% for isoprenaline, 163% and 157% for lipolysis produced by isoprenaline in the presence of EPA and DHA respectively) (fig. 4.38).

The results presented in figs. 4.39(a) and (b), and 4.40(a) and (b) show the inhibition by EPA and DHA of adenylate cyclase and lipolytic activity induced by a range of stimuli. A significant inhibition of adenylate cyclase and lipolytic activity due to isoprenaline and forskolin was observed with EPA and DHA. In addition to this, EPA and DHA inhibited lipolysis due to dibutyryl cAMP.

An investigation into the effect of increasing the pH value of the vehicle used for the fatty acids was designed to assess the importance of the degree of ionization of EPA and DHA as regards their inhibitory effect on adenylate cyclase and to determine whether the conversion of any free fatty acid which may possibly remain in the incubation mixture under previous experimental conditions to a salt affects the inhibitory action of EPA and DHA on adenylate cyclase activity. The results presented in fig. 4.41 revealed that increasing the pH of the vehicle did not prevent EPA or DHA from inhibiting isoprenaline-stimulated adenylate cyclase

Figure 4.27(a)

Effect of Eicosapentaenoic Acid on cAMP Production with 50μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + EPA (83μM)

4 = isoprenaline (1μM) + EPA (165μM)

5 = isoprenaline (1μM) + EPA (248μM)

6 = isoprenaline (1μM) + EPA (331μM)

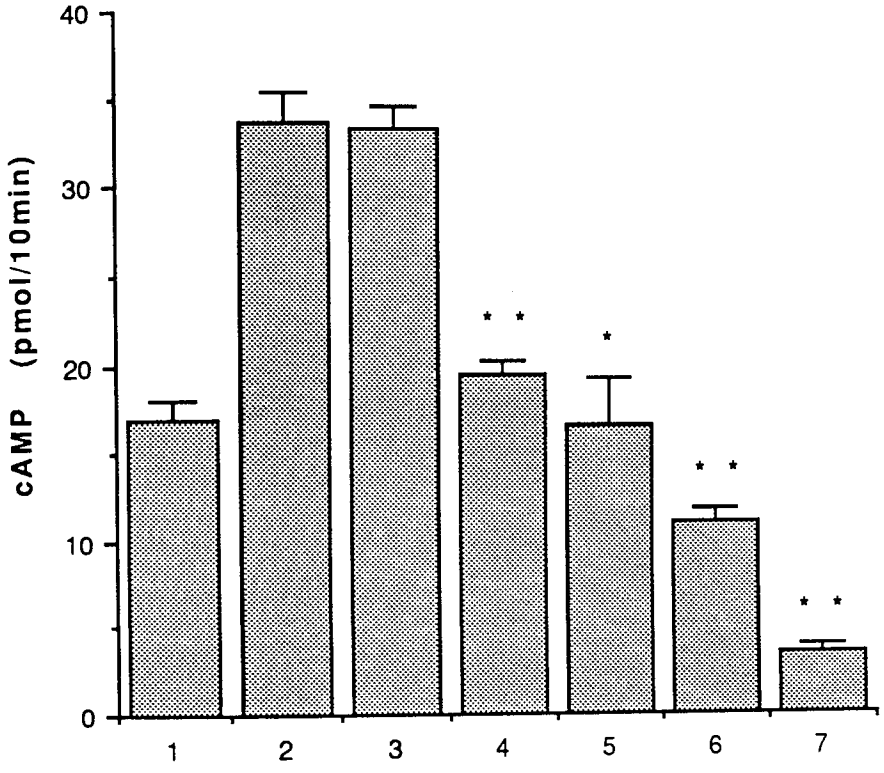
7 = isoprenaline (1μM) + EPA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments.

*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 (compared with iso, STT).

Figure 4.27(b)

Effect of Docosahexaenoic Acid on cAMP Production with 50μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + DHA (83μM)

4 = isoprenaline (1μM) + DHA (165μM)

5 = isoprenaline (1μM) + DHA (248μM)

6 = isoprenaline (1μM) + DHA (331μM)

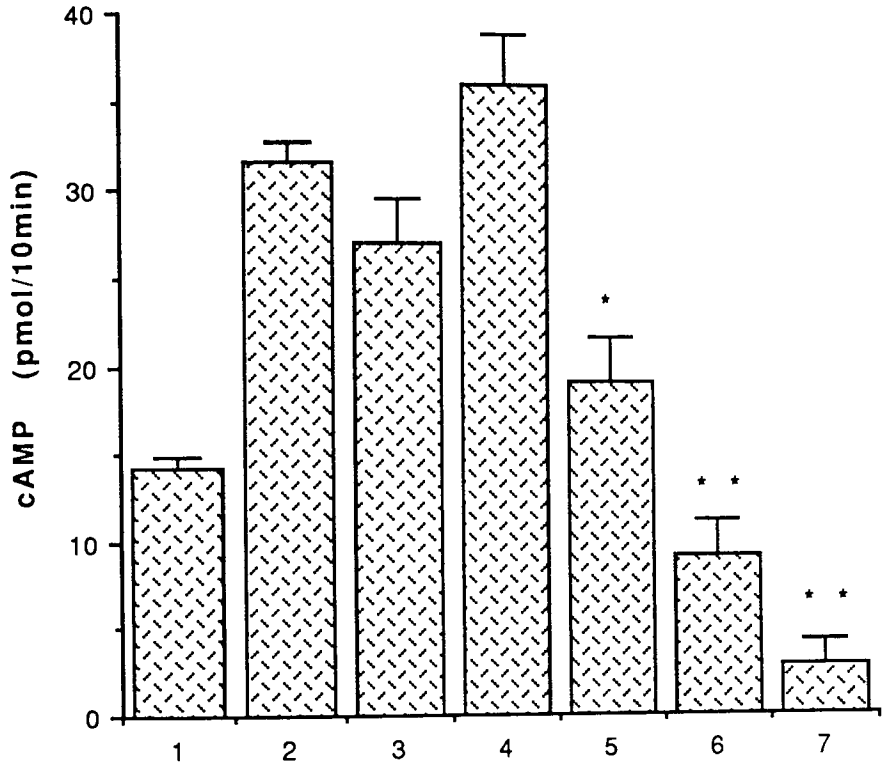
7 = isoprenaline (1μM) + DHA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments.

*p < 0.005, **p < 0.001 (compared with iso, STT).

Figure 4.27(c)

Effect of Arachidonic Acid on cAMP Production with 50μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + AA (83μM)

4 = isoprenaline (1μM) + AA (165μM)

5 = isoprenaline (1μM) + AA (248μM)

6 = isoprenaline (1μM) + AA (331μM)

7 = isoprenaline (1μM) + AA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments.

*p < 0.01, **p < 0.001 (compared with iso, STT).

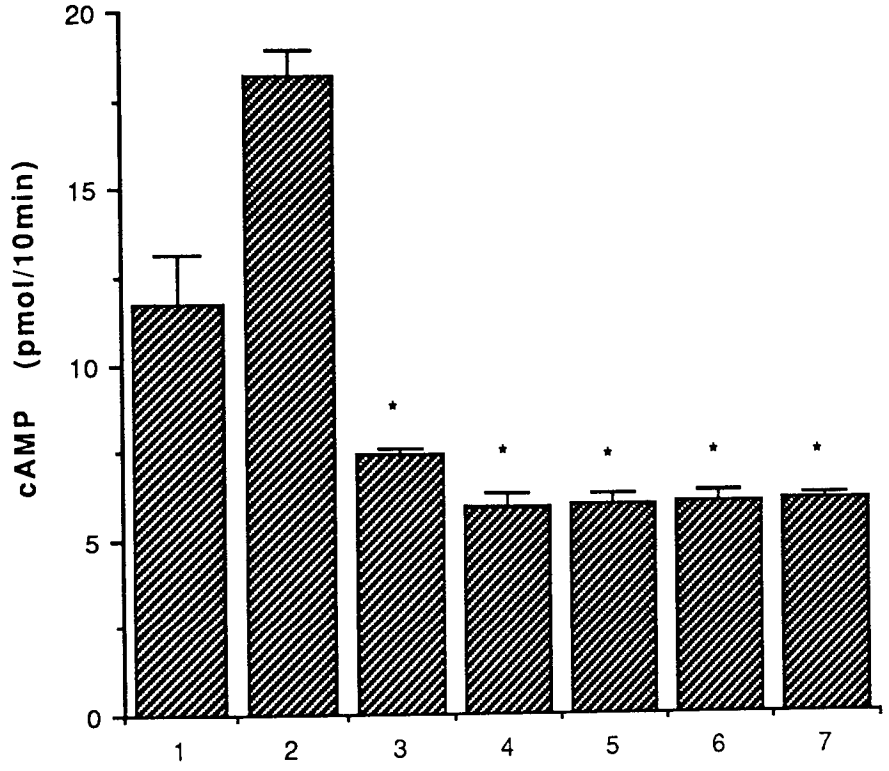
activity. However, only EPA retained the dose-dependent inhibition of adenylate cyclase activity. DHA inhibited activity to a similar extent as EPA with 248μM fatty acid, but to a significantly lesser extent at a concentration of 496μM.

4.3.1.2.3.3 Discussion

When 100μg adipocyte plasma membrane protein was incubated with EPA, DHA or AA, an increase in isoprenaline-stimulated cAMP production was observed with all three fatty acids. In contrast, with 50μg membrane protein a concentration-dependent inhibition of cAMP production was observed with EPA, DHA and AA. Having obtained these two conflicting results, the effect of the three fatty acids on isoprenaline-stimulated cAMP production was investigated with a wide range of membrane protein concentrations. These investigations suggested that EPA, DHA and AA produce two opposing effects on adenylate cyclase activity. Stimulation appears to occur with low fatty acid : membrane protein ratios and inhibition predominates with high ratios of fatty acid : membrane protein. The point at which the stimulatory and inhibitory effects of EPA and DHA appear to cancel one another out is at a membrane concentration of around 75μg/100μl. Arachidonic acid, however, continues to stimulate adenylate cyclase activity significantly at this membrane concentration, indicating a difference between this ω-6 fatty

Figure 4.28(a)

Effect of Eicosapentaenoic Acid on cAMP Production with 25μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + EPA (83μM)

4 = isoprenaline (1μM) + EPA (165μM)

5 = isoprenaline (1μM) + EPA (248μM)

6 = isoprenaline (1μM) + EPA (331μM)

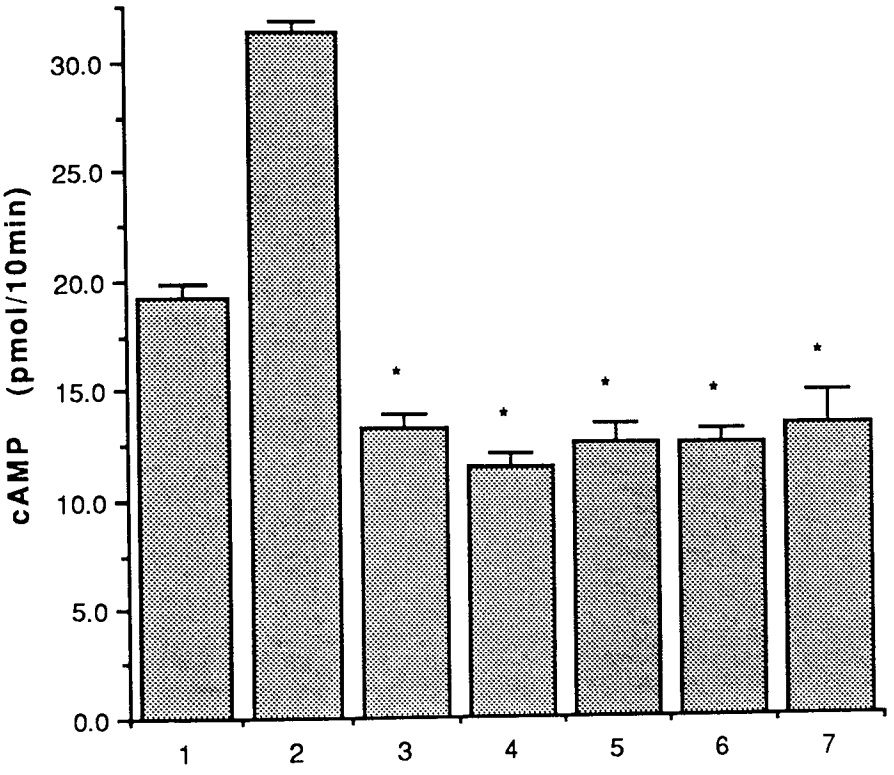
7 = isoprenaline (1μM) + EPA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments

*p < 0.001 (compared with iso, STT).

Figure 4.28(b)

Effect of Docosahexaenoic Acid on cAMP Production with 25μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + DHA (83μM)

4 = isoprenaline (1μM) + DHA (165μM)

5 = isoprenaline (1μM) + DHA (248μM)

6 = isoprenaline (1μM) + DHA (331μM)

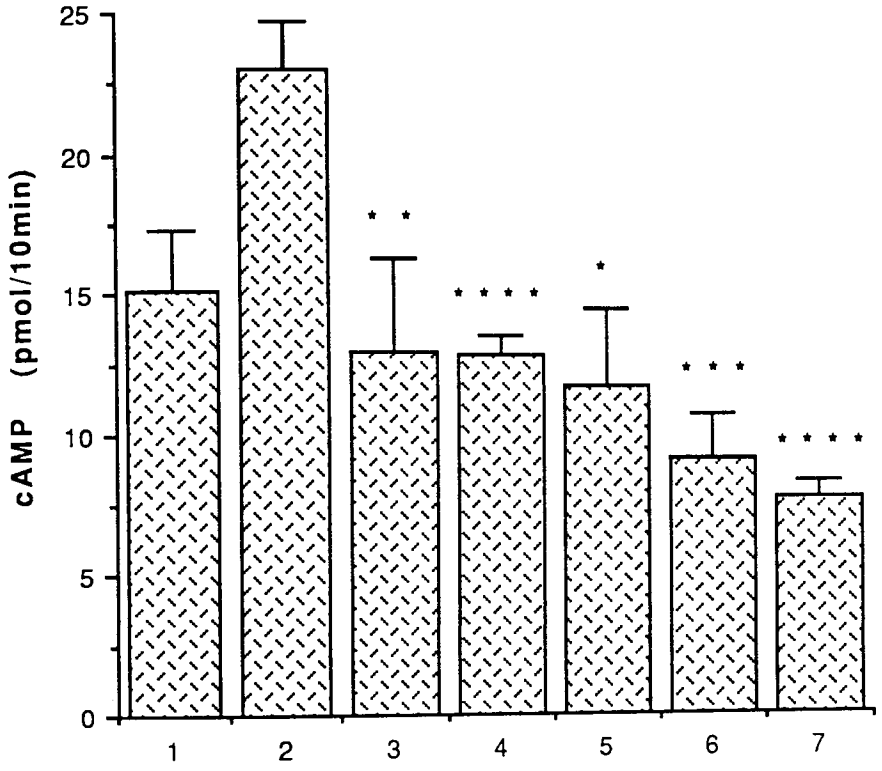
7 = isoprenaline (1μM) + DHA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments.

*p < 0.001 (compared with iso, STT).

Figure 4.28(c)

Effect of Arachidonic Acid on cAMP Production with 25µg Plasma Membranes



1 = basal

2 = isoprenaline (1µM)

3 = isoprenaline (1µM) + AA (83µM)

4 = isoprenaline (1µM) + AA (165µM)

5 = isoprenaline (1µM) + AA (248µM)

6 = isoprenaline (1µM) + AA (331µM)

7 = isoprenaline (1µM) + AA (496µM)

Results are expressed as mean ± SEM of 3 - 4 experiments.

*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 (compared with iso, STT).

acid and the two ω-3 fatty acids.

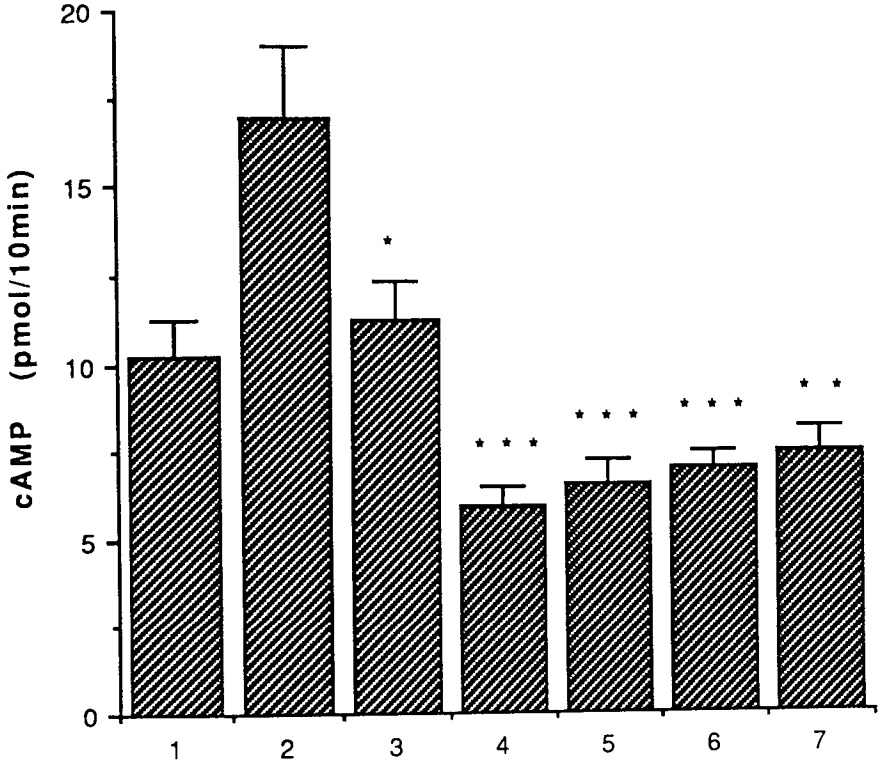
The dual action of EPA, DHA and AA observed in the present study is reminiscent of the effect of these fatty acids on ADP-ribosylation catalysed by pertussis toxin (see Section 4.3.1.2.2). This suggests that the three fatty acids may be acting via G_i to regulate isoprenaline-stimulated adenylate cyclase activity.

An examination of the time course for stimulation/inhibition of adenylate cyclase activity activated by isoprenaline revealed that the degree of stimulation remained approximately constant at early time points and increased with increasing incubation time. The inhibitory effect of EPA also remained constant at early time points, the rate of cAMP production due to isoprenaline being greater in the absence of EPA during this time period. The rates of isoprenaline-stimulated adenylate cyclase activity in the presence and absence of EPA became similar with increasing incubation time when isoprenaline-stimulated cAMP production reached saturation point.

Varying the isoprenaline concentration showed that EPA enhanced basal adenylate cyclase activity with 100μg adipocyte plasma membranes. This appears to be the main effect of EPA at this membrane concentration and probably accounts for the rise in adenylate cyclase activity observed with isoprenaline concentrations from 0 - 0.4μM. The percentage stimulation of adenylate cyclase activity decreases with higher isoprenaline concentrations which indicates that there

Figure 4.29(a)

Effect of Eicosapentaenoic Acid on cAMP Production with 10μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + EPA (83μM)

4 = isoprenaline (1μM) + EPA (165μM)

5 = isoprenaline (1μM) + EPA (248μM)

6 = isoprenaline (1μM) + EPA (331μM)

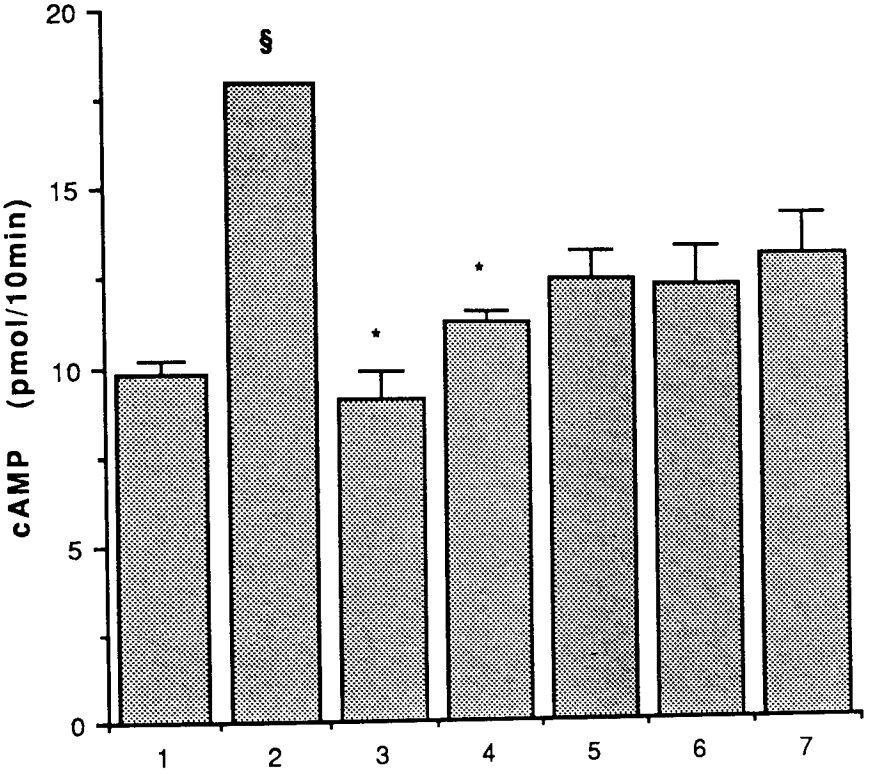
7 = isoprenaline (1μM) + EPA (496μM)

Results are expressed as mean ± SEM of 3 experiments.

*p < 0.05, **p < 0.01, ***p < 0.005 (compared with iso, STT).

Figure 4.29(b)

Effect of Docosahexaenoic Acid on cAMP Production with 10μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + DHA (83μM)

4 = isoprenaline (1μM) + DHA (165μM)

5 = isoprenaline (1μM) + DHA (248μM)

6 = isoprenaline (1μM) + DHA (331μM)

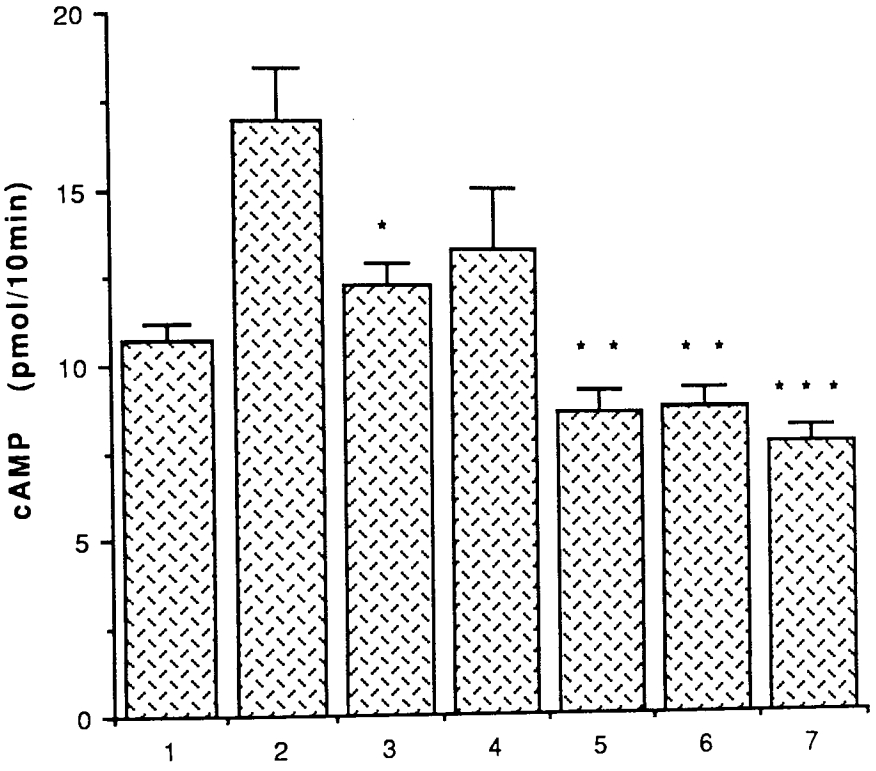
7 = isoprenaline (1μM) + DHA (496μM)

Results are expressed as mean ± SEM of 3 experiments. § = mean of 2 experiments.

*$p < 0.05$ (compared with iso, STT).

Figure 4.29(c)

Effect of Arachidonic Acid on cAMP Production with 10μg Plasma Membranes



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + AA (83μM)

4 = isoprenaline (1μM) + AA (165μM)

5 = isoprenaline (1μM) + AA (248μM)

6 = isoprenaline (1μM) + AA (331μM)

7 = isoprenaline (1μM) + AA (496μM)

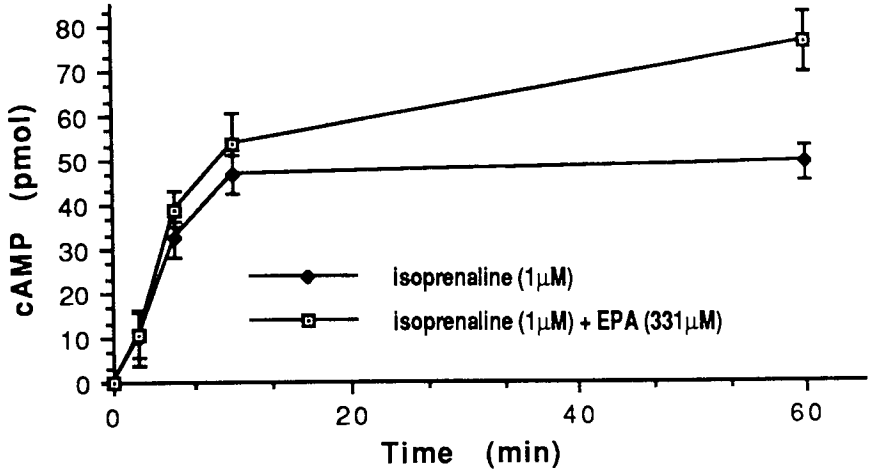
Results are expressed as mean ± SEM of 3 - 4 experiments. *p < 0.05, **p < 0.005, ***p < 0.001 (compared with iso, STT).

may be a "fine tuning" effect on the modulation of adenylate cyclase activity by EPA which is affected by the agonist concentration. The degree of inhibition of adenylate cyclase activity was increased at high concentrations of isoprenaline. These results suggest that the concentration of the agonist is also an important factor in the regulation of adenylate cyclase activity by EPA, and that the inhibitory effect of EPA is favoured at high agonist concentrations. The importance of agonist concentration on the modulatory effects of polyunsaturated fatty acids may also account for the observation that EPA, DHA and AA inhibit adenylate cyclase activity at a concentration of 248μM fatty acid with 50μg membrane protein, yet they stimulate activity at a fatty acid concentration of 496μM with 100μg membrane protein. These two preparations have the same ratio of fatty acid : plasma membrane but the ratio of isoprenaline : plasma membrane is doubled with 50μg plasma membranes since the isoprenaline concentration was kept constant at 1μM with varying membrane concentration.

The results so far suggest that EPA, DHA and AA may interact with G_i and/or G_s to modulate adenylate cyclase activity. An interaction of these fatty acids with G_i was determined in Section 4.3.1.2.2, in addition to which the possibility of an interaction of the fatty acids with G_s was suggested. The present results may indicate that EPA exhibits dual regulation of G_s, activating this G-protein when it is in a state of low activity and inactivating the highly active form

Figure 4.30

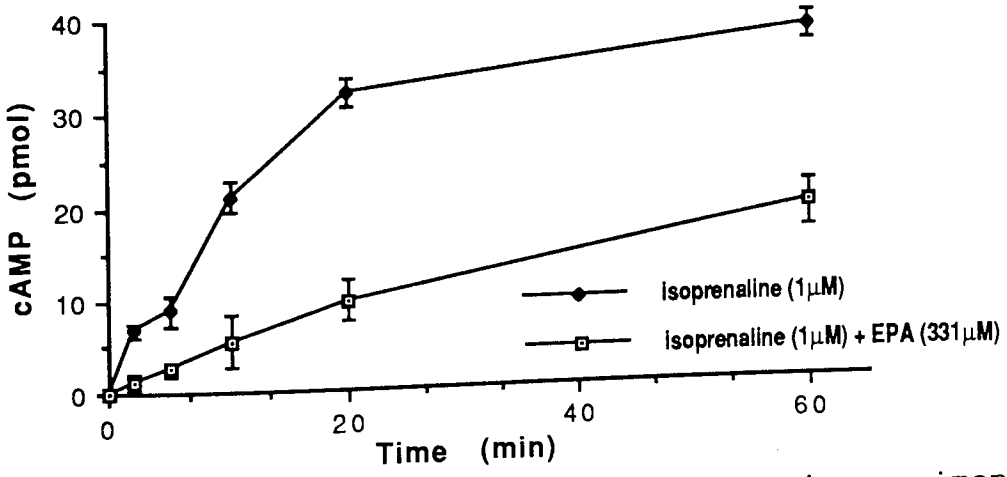
Time Course for Stimulation of Adenylate Cyclase by EPA with 100μg Plasma Membranes



Results are expressed as mean ± SEM of 3 - 4 experiments.
Basal adenylate cyclase activity = 15.2 ± 1.4 pmol cAMP/10 min (mean ± SEM of 3 experiments)

Figure 4.31

Time Course for Inhibition of Adenylate Cyclase by EPA with 50μg Plasma Membranes

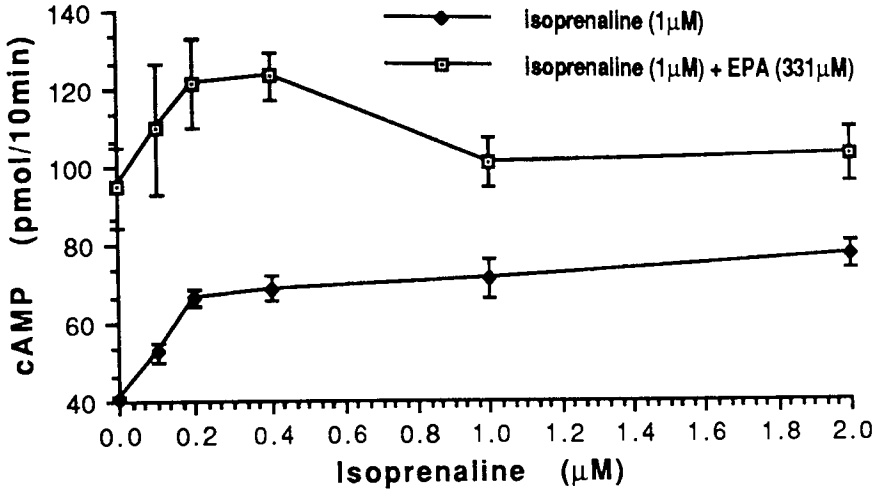


Results are expressed as mean ± SEM of 3 - 4 experiments.
Basal adenylate cyclase activity = 14.2 ± 0.6 pmol cAMP/10 min (mean ± SEM of 3 experiments).

of G_s. This is a possible modulatory role of EPA on adenylate cyclase activity but more detailed studies are required before any definite conclusion can be made regarding the involvement of G_s in the modulation of adenylate cyclase activity by EPA and DHA. Orly and Schramm (1975) suggested that an observed increase in isoprenaline-stimulated adenylate cyclase activity after treatment of turkey erythrocyte plasma membranes with specific fatty acids was due to increased introduction of GTP to the regulatory component of isoprenaline-stimulated adenylate cyclase (now known to be G_s). Unsaturated fatty acids, which were likely to cause an increase in membrane fluidity (Fernandes et al, 1989) were the most potent in enhancing hormonal activation of adenylate cyclase. Orly and Schramm (1975) suggested that phospholipids may be required for the functional introduction of GTP to its binding site and that certain fatty acids may facilitate this process. Following this explanation, a possible mechanism for the dual action of polyunsaturated fatty acids on adenylate cyclase function observed in the present study may be as follows. At relatively low fatty acid : plasma membrane ratios, as seen in the study by Orly and Schramm and with 100µg plasma membranes in the present study, certain fatty acids facilitate the introduction of GTP to G_s and hence increase the activation of adenylate cyclase. Increasing the fatty acid : plasma membrane ratio may cause a further alteration in membrane composition which inhibits the introduction of GTP to G_s and therefore, in addition to inactivating G_s, favours the

Figure 4.32

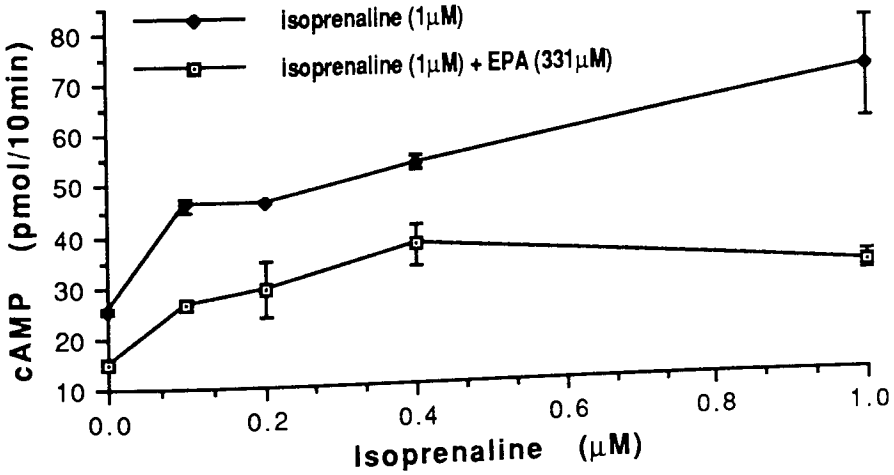
Effect of Varying Isoprenaline Concentration on Stimulation of Adenylate Cyclase by EPA with 100μg Plasma Membranes



Results are expressed as mean ± SEM of 3 experiments.

Figure 4.33

Effect of Varying Isoprenaline Concentration on Inhibition of Adenylate Cyclase by EPA with 50μg Plasma Membranes



Results are expressed as mean ± SEM of 3 experiments.

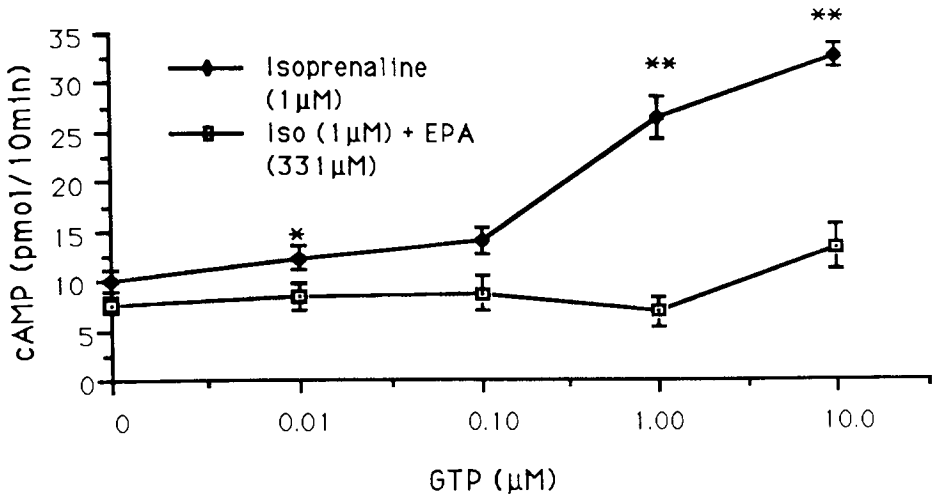
introduction of GTP to G_i, thus activating this G-protein. The functional consequences of the hypothesized interaction of EPA, DHA and AA with G_s may be modulated by the state of activity of this G-protein, as shown by the effects of varying agonist concentration. However, this is not as important a factor as the ratio of fatty acid to plasma membrane concentration as regards the effect of the fatty acids on adenylate cyclase activity.

An investigation into the effect of increasing GTP concentration on the inhibition of isoprenaline-stimulated adenylate cyclase activity using 50µg plasma membranes further supports the suggestion that G_i is involved in the inhibitory mechanism of EPA and DHA. Although the level of adenylate cyclase activity remained constant and did not decrease with increasing GTP concentration in EPA- and DHA-treated membranes, the degree of inhibition produced by these fatty acids increased significantly compared with isoprenaline-stimulated activity as GTP concentrations increased. However, the present investigation revealed an additional effect of the fatty acids studied. Using 50µg plasma membranes, stimulation of adenylate cyclase activity with isoprenaline was inhibited by EPA and DHA with no GTP added to the incubation mixture. This is an indication that the inhibitory effect observed is, at least in part, due to a direct effect of the fatty acids on the catalytic unit of adenylate cyclase.

In order to study the link between alterations in

Figure 4.34(a)

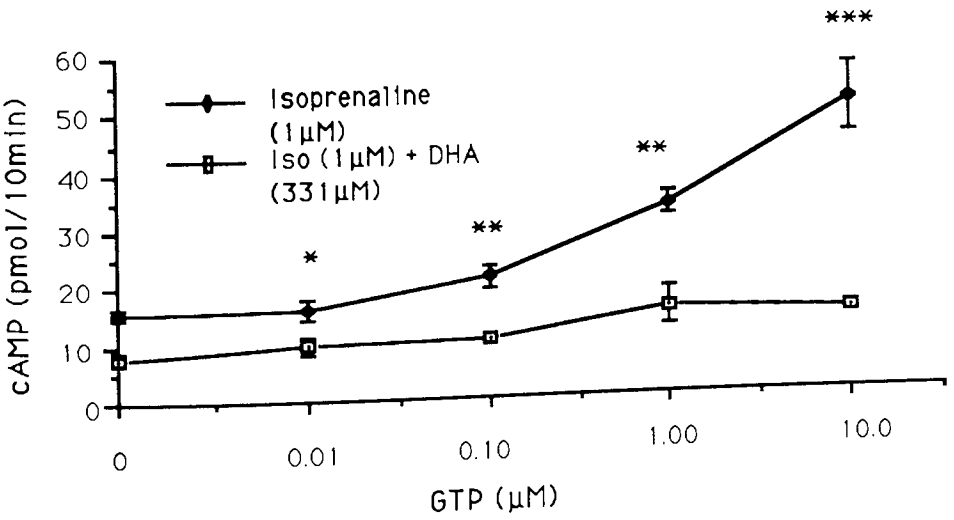
Effect of Varying GTP Concentration on Inhibition of Adenylate Cyclase by Eicosapentaenoic Acid



Results are expressed as mean ± SEM of 3 experiments.
Membrane concentration = 50μg/100μl.
*p < 0.05, **p < 0.001 (iso + EPA compared with iso, STT).

Figure 4.34(b)

Effect of Varying GTP Concentration on Inhibition of Adenylate Cyclase by Docosahexaenoic Acid

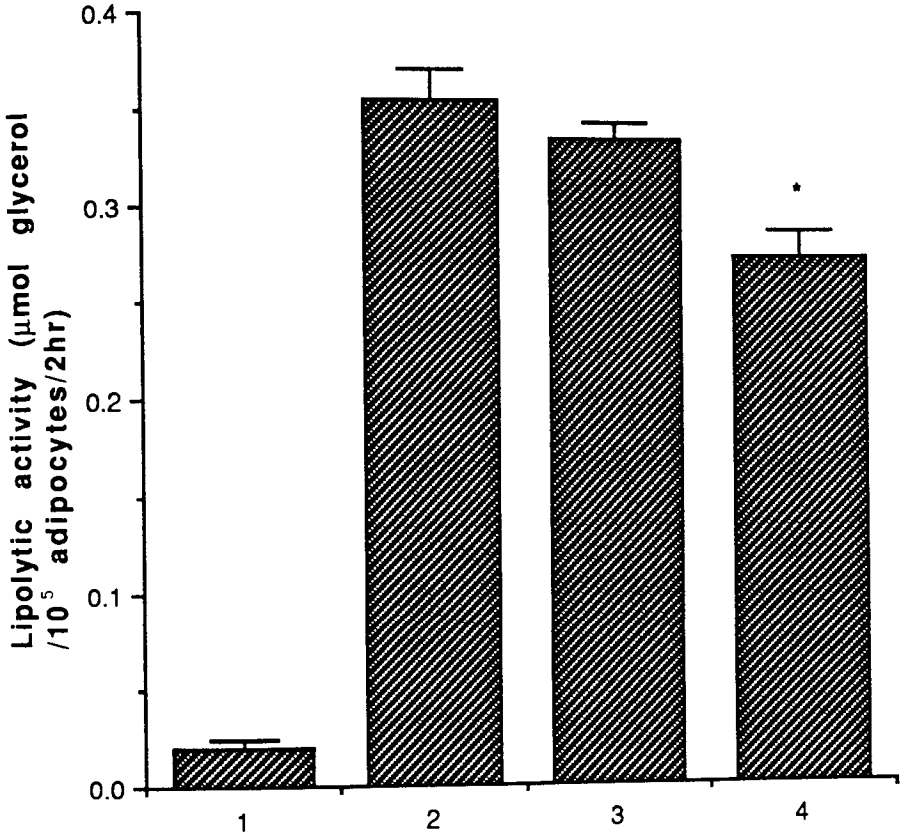


Results are expressed as mean ± SEM of 3 - 4 experiments.
Membrane concentration = 50μg/100μl.
*p < 0.05, **p < 0.005, ***p < 0.001 (iso + DHA compared with iso, STT).

adenylate cyclase activity and lipolysis, the effect of EPA, DHA and AA on isoprenaline-stimulated lipolytic activity in rat adipocytes was investigated. All three fatty acids produced a degree of inhibition of lipolysis. The inhibition produced by EPA and DHA was significant ($p < 0.005$ at 496μM) and dose-related. Arachidonic acid inhibited lipolysis to a lesser extent ($p < 0.05$) and was not dose-related. These results correlated fairly well with those from earlier studies in this Section in that they showed almost identical responses with the ω-3 fatty acids, EPA and DHA, and a slightly different response with the ω-6 fatty acid, arachidonic acid. The anti-lipolytic effect of DHA observed in the present study had previously been reported to be absent (Tisdale and Beck, 1991). This may have been due to the fact that the methyl ester of DHA was used in the study quoted above, which may not be so readily incorporated into the plasma membrane. This result suggests that insertion of these fatty acids into the membrane is necessary for their effect on adenylate cyclase and lipolytic activity. The results showing the extent of incorporation of EPA into adipocyte plasma membranes indicate that EPA, after incubation with membranes for 15 min at 37°C, is associated with the outside of the membrane, in addition to being incorporated within the membrane. It is not known into which membrane lipids EPA was incorporated, if indeed it is incorporated into membrane lipids *in vitro*, but previous studies (Sakaguchi *et al*, 1990) have shown preferential incorporation into phospholipid fractions. The observation

Figure 4.35(a)

Effect of Eicosapentaenoic Acid on Isoprenaline-Stimulated Lipolysis in Rat Adipocytes



1 = fat cells

2 = fat cells + isoprenaline (1µM)

3 = fat cells + isoprenaline (1µM) + EPA (248µM)

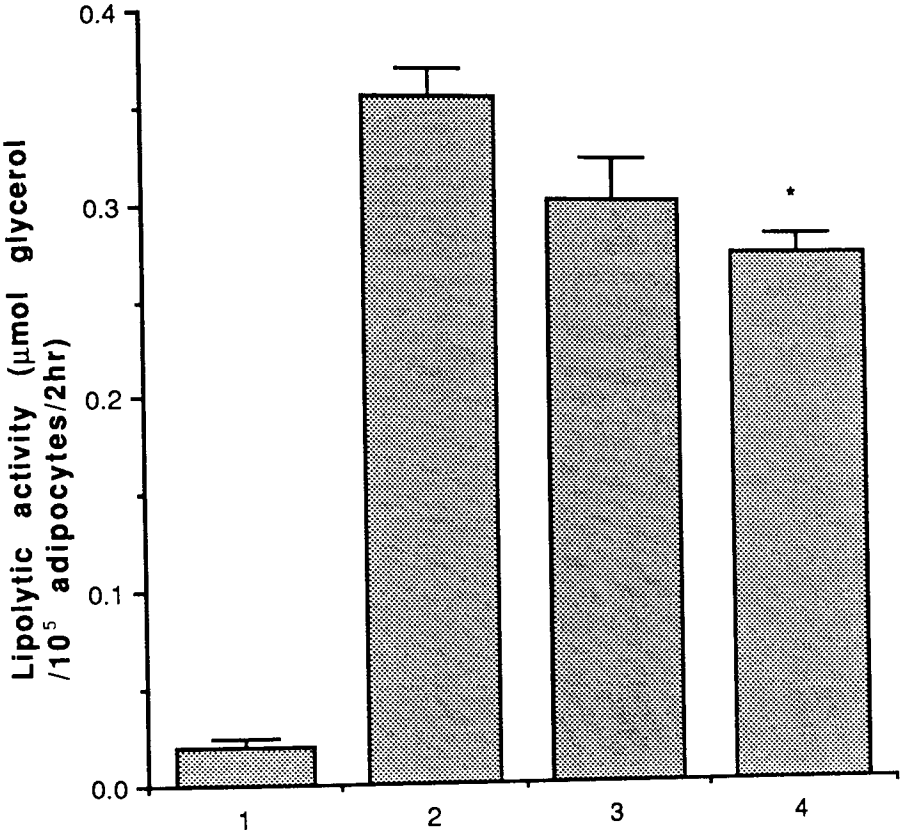
4 = fat cells + isoprenaline (1µM) + EPA (496µM)

Results are expressed as mean ± SEM of 4 experiments.

*$p < 0.005$ (compared with iso, STT).

Figure 4.35(b)

Effect of Docosahexaenoic Acid on Isoprenaline-Stimulated Lipolysis in Rat Adipocytes



1 = fat cells

2 = fat cells + isoprenaline (1µM)

3 = fat cells + isoprenaline (1µM) + DHA (248µM)

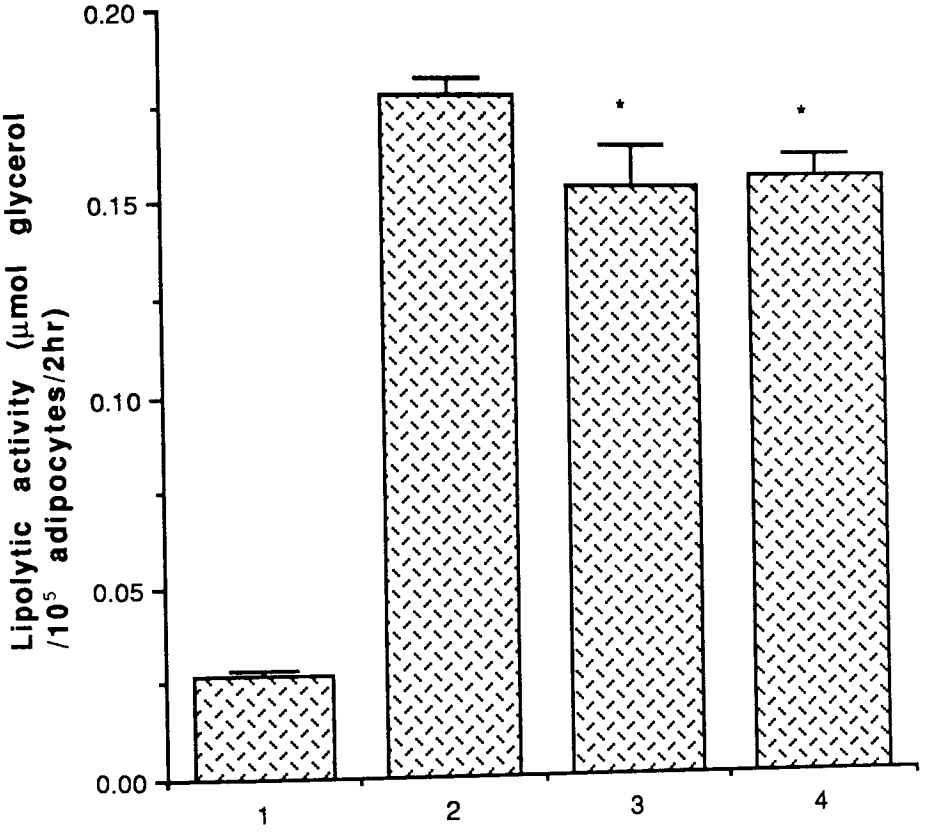
4 = fat cells + isoprenaline (1µM) + DHA (496µM)

Results are expressed as mean ± SEM of 4 experiments.

*$p < 0.005$ (compared with iso, STT).

Figure 4.35(c)

Effect of Arachidonic Acid on Isoprenaline-Stimulated Lipolysis in Rat Adipocytes



1 = fat cells

2 = fat cells + isoprenaline (1µM)

3 = fat cells + isoprenaline (1µM) + AA (248µM)

4 = fat cells + isoprenaline (1µM) + AA (496µM)

Results are expressed as mean ± SEM of 4 experiments.

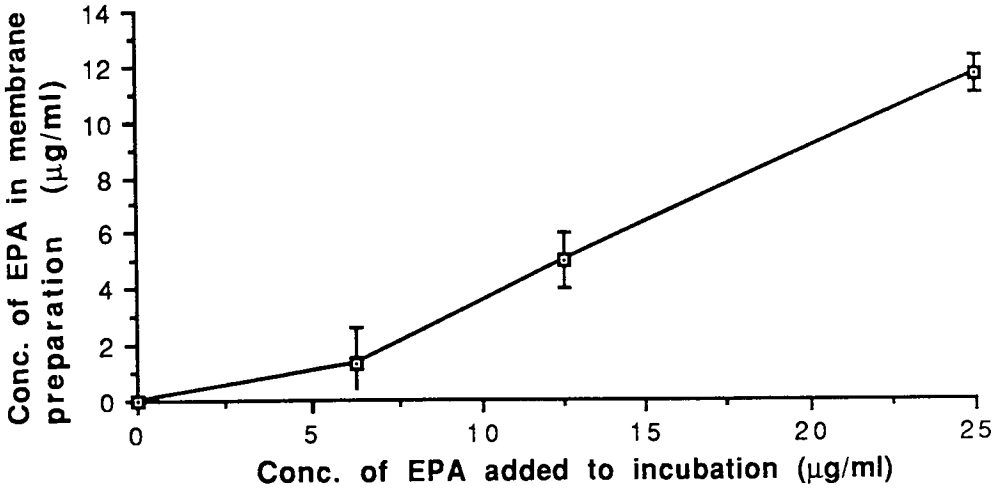
*$p < 0.05$ (compared with iso, STT).

that EPA was associated with the adipocyte plasma membrane both internally and externally, suggests that it may produce physiological alterations as a result of an interaction with an external membrane receptor, and/or by replacing endogenous fatty acids in membrane lipids and altering membrane fluidity. Increased erythrocyte plasma membrane fluidity as a result of a fish oil diet has been noted by Fernandes et al (1989).

Pertussis toxin treatment has been shown to inactivate G_i (the mechanism of this inactivation is discussed in detail in Section 1.5.3.2) and reverses hormonal inhibition of lipolysis (eg. Valet et al, 1990). This toxin would therefore give some indication as to whether EPA, DHA and AA produce their inhibitory effect via a membrane receptor. Following the indications from previous experiments in this section that G_i may be involved in the inhibitory mechanism of EPA, DHA and AA on adenylate cyclase activity, this toxin was employed as a tool for determining whether the proposed interaction with G_i has functional consequences on these processes. Pertussis toxin treatment did not completely abolish the inhibitory effect of the fatty acids on adenylate cyclase activity or lipolysis. Although the difference between pertussis toxin-treated and untreated membranes was similar in basal/isoprenaline-stimulated samples and fatty acid - treated samples, the percentage increase in activity due to pertussis toxin treatment was greater in the fatty acid - treated samples. This was not seen, however, with the results of the lipolysis assay in which the percentage increase in

Figure 4.36(a)

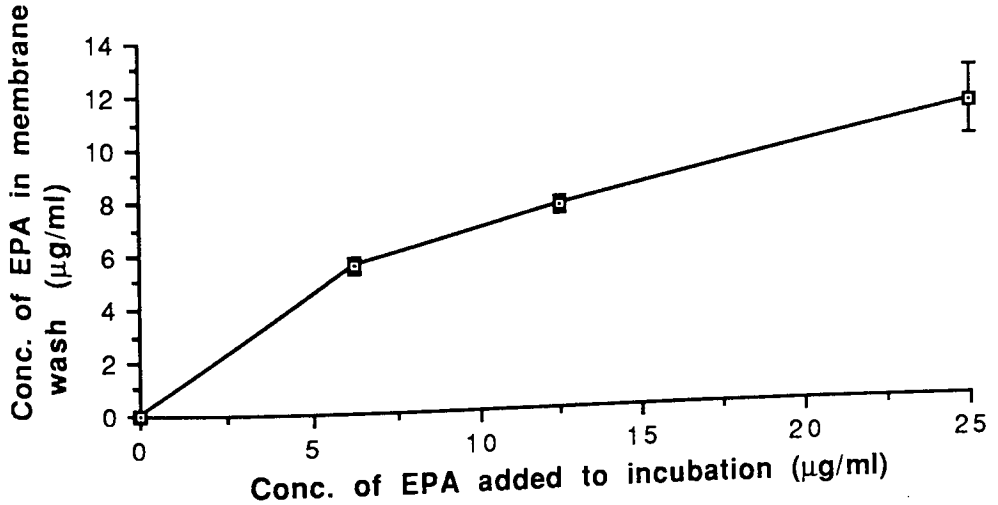
Uptake of Eicosapentaenoic Acid Into Adipocyte Membrane Preparations



Results are expressed as mean ± SEM of 4 experiments.
Membrane concentration = 50µg/100µl.

Figure 4.36(b)

Eicosapentaenoic Acid Externally Associated With Adipocyte Membrane



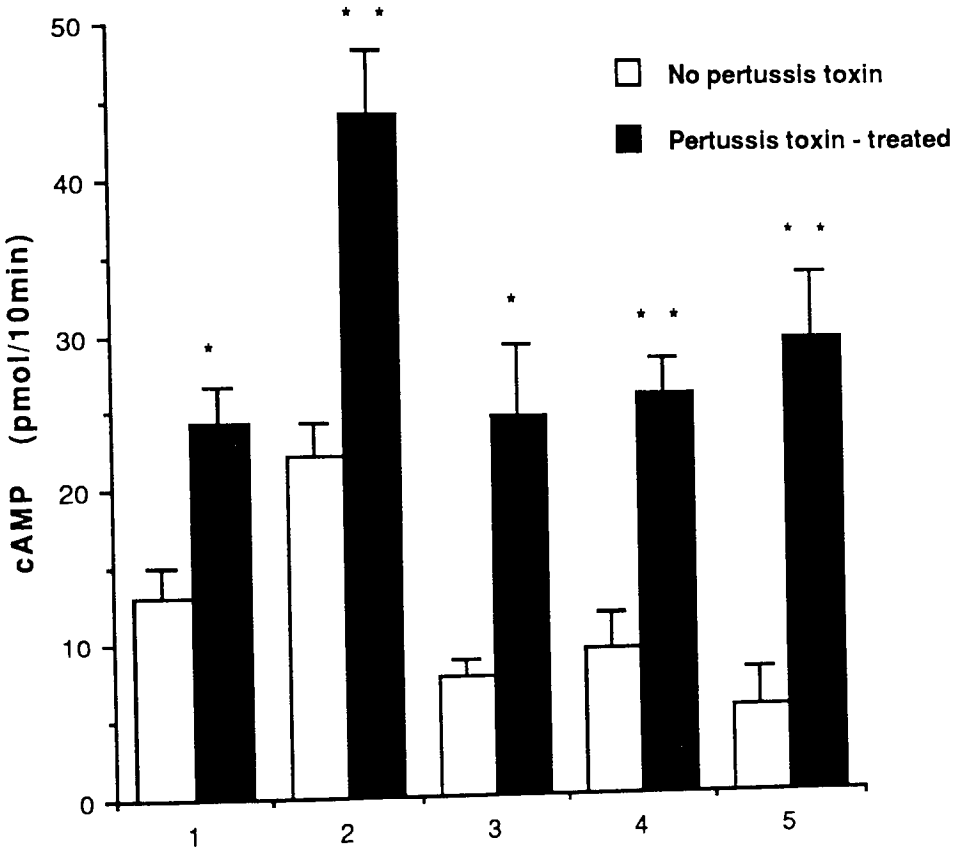
Results are expressed as mean ± SEM of 4 experiments.
Membrane concentration = 50µg/100µl.

activity due to pertussis toxin - treatment was only slightly higher in fatty acid - treated samples than that of untreated samples when stimulated with isoprenaline. Pertussis toxin treatment is thought to uncouple inhibitory receptors from G_i (eg. Cote et al, 1984) and therefore prevent inhibition of receptor-stimulated G_i-mediated intracellular events. The results of this experiment therefore suggest that the effect of the fatty acids studied is not consistent with hormonal inhibition. The small effect of pertussis toxin treatment on fatty acid - induced inhibition of adenylate cyclase activity was of doubtful significance since it was not reflected in the study of their anti-lipolytic action.

In an attempt to further characterize, and perhaps differentiate between, the mechanisms of inhibition of adenylate cyclase and lipolysis by EPA and DHA, an examination was made of the effect of the fatty acids on the stimulation of these two processes. Both fatty acids were found to significantly inhibit the increased adenylate cyclase activity and lipolysis induced by isoprenaline and forskolin. Forskolin stimulates the catalytic unit of adenylate cyclase, and inhibition of cAMP production by this agonist was expected since it has already been suggested that EPA and DHA may act directly and/or indirectly on this catalytic unit. Dibutyryl cAMP introduces a new dimension to the mechanism of lipolysis. This lipolytic agent, a cAMP analogue, is not susceptible to hydrolysis by phosphodiesterase (Jarett and Smith, 1974). The decrease in cAMP breakdown due to competitive inhibition of

Figure 4.37

Effect of Pertussis Toxin Treatment on the Inhibition of Adenylate Cyclase by Eicosapentaenoic, Docosahexaenoic and Arachidonic Acids



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + EPA (496μM)

4 = isoprenaline (1μM) + DHA (496μM)

5 = isoprenaline (1μM) + AA (496μM)

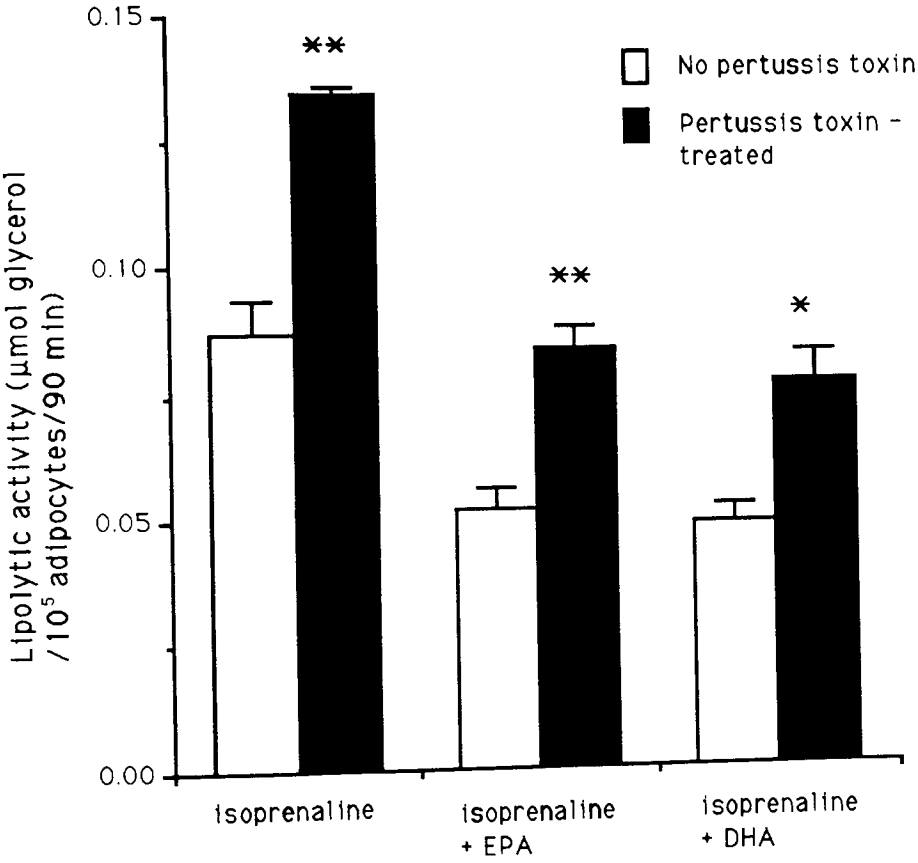
Results are expressed as mean ± SEM of 3 - 4 experiments.

Membrane concentration = 50μg/100μl.

*p < 0.05, **p < 0.01 (PT-treated compared with untreated membranes, STT).

Figure 4.38

Effect of Pertussis Toxin on the Inhibition of Lipolysis by Eicosapentaenoic and Docosahexaenoic Acids



Results are expressed as mean ± SEM of 4 experiments.

Isoprenaline concentration = 1 µM.

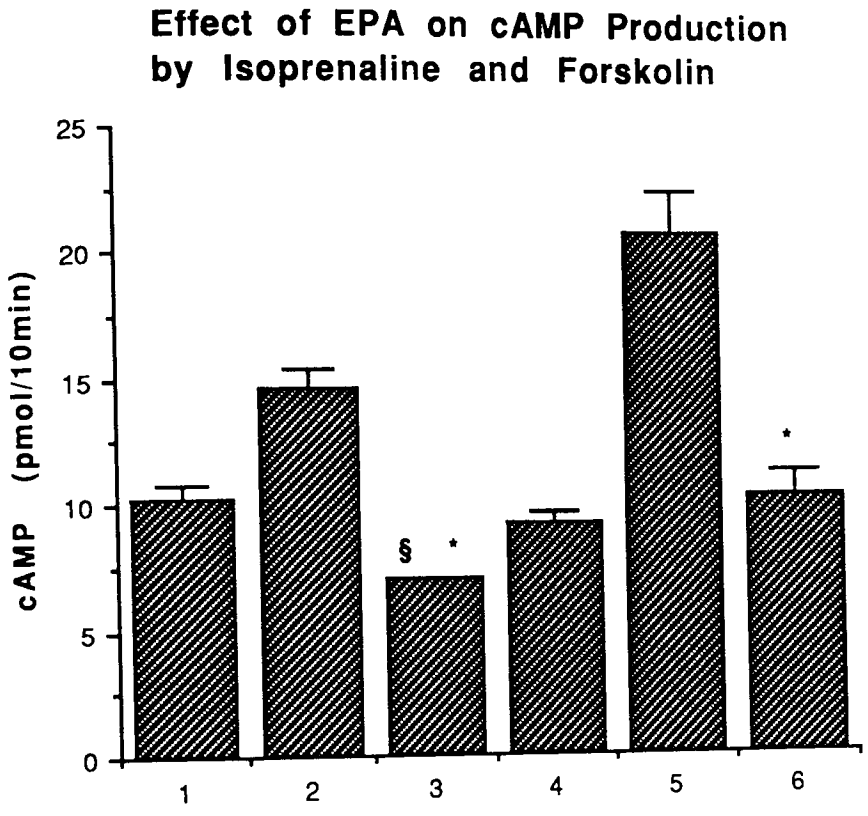
Fatty acid concentration = 496µM.

*p < 0.005, **p < 0.001 (PT-treated compared with untreated cells, STT).

phosphodiesterase leads to an increase in lipolysis. EPA and DHA significantly inhibited lipolysis due to dibutyryl cAMP. If these fatty acids were to exert their anti-lipolytic effect by activating phosphodiesterase, no decrease in lipolysis would be observed after the inactivation of this enzyme by dibutyryl cAMP. Since dibutyryl cAMP competes directly with cAMP for phosphodiesterase, it is unlikely that EPA and DHA selectively alter the interaction of dibutyryl cAMP with phosphodiesterase leading to increased cAMP breakdown and decreased lipolysis. The most likely explanation is that the observed decrease in lipolysis is due to the decrease in catalytic adenylate cyclase activity observed with EPA and DHA, leading to a reduction in basal cAMP production and accumulation over the time period studied.

In all previous experiments, fatty acids were dissolved in either equimolar lysine or ethanol. In order to attempt to differentiate between the actions of EPA and DHA, which so far appear to be identical, the pH of the vehicle for the fatty acids was increased using an excess of lysine plus sodium carbonate to remove any free fatty acid which may have remained when EPA and DHA were dissolved in lysine. EPA inhibited isoprenaline-stimulated adenylate cyclase activity in the same dose-related manner with equimolar lysine or lysine/Na_2CO_3 but the inhibition due to DHA lost its dose-dependency when the pH of the vehicle was increased. This suggests that the degree of ionization of this fatty acid may be important in the mechanism of its inhibitory effect on

Figure 4.39(a)



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + EPA (331μM)

4 = DMSO

5 = forskolin (25μM)

6 = forskolin (25μM) + EPA (331μM)

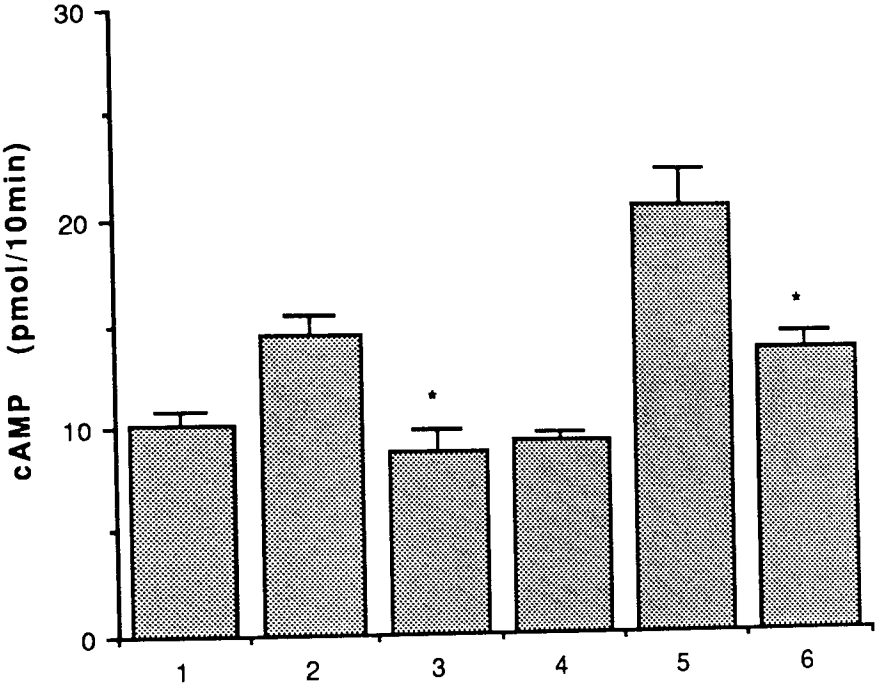
Results are expressed as mean ± SEM of 4 experiments. § = mean of 2 experiments.

Membrane concentration = 50μg/100μl.

*p < 0.001 (compared with agonist, STT).

Figure 4.39(b)

Effect of DHA on cAMP Production by Isoprenaline and Forskolin



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + DHA (331μM)

4 = DMSO

5 = forskolin (25μM)

6 = forskolin (25μM) + DHA (331μM)

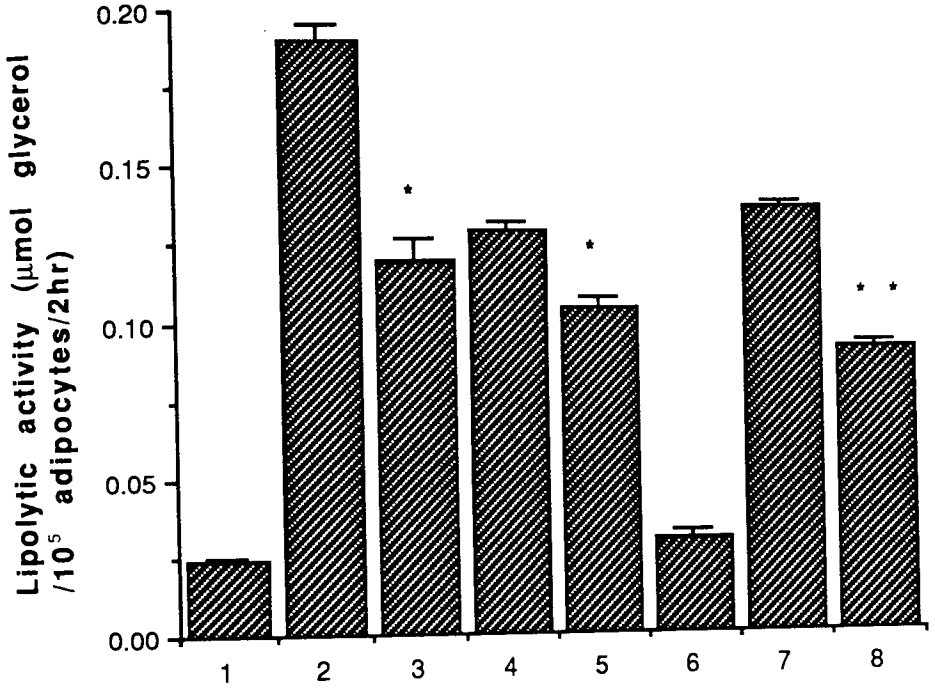
Results are expressed as mean ± SEM of 3 - 4 experiments.

Membrane concentration = 50μg/100μl.

*p < 0.005 (compared with agonist, STT).

Figure 4.40(a)

Effect of EPA on Lipolytic Activity Produced by Isoprenaline, Dibutyryl cAMP and Forskolin in Mouse Adipocytes



1 = fat cells

2 = fat cells + isoprenaline (1µM)

3 = fat cells + isoprenaline (1µM) + EPA (331µM)

4 = fat cells + dibutyryl cAMP (1mM)

5 = fat cells + dibutyryl cAMP (1mM) + EPA (331µM)

6 = fat cells + DMSO

7 = fat cells + forskolin (25µM)

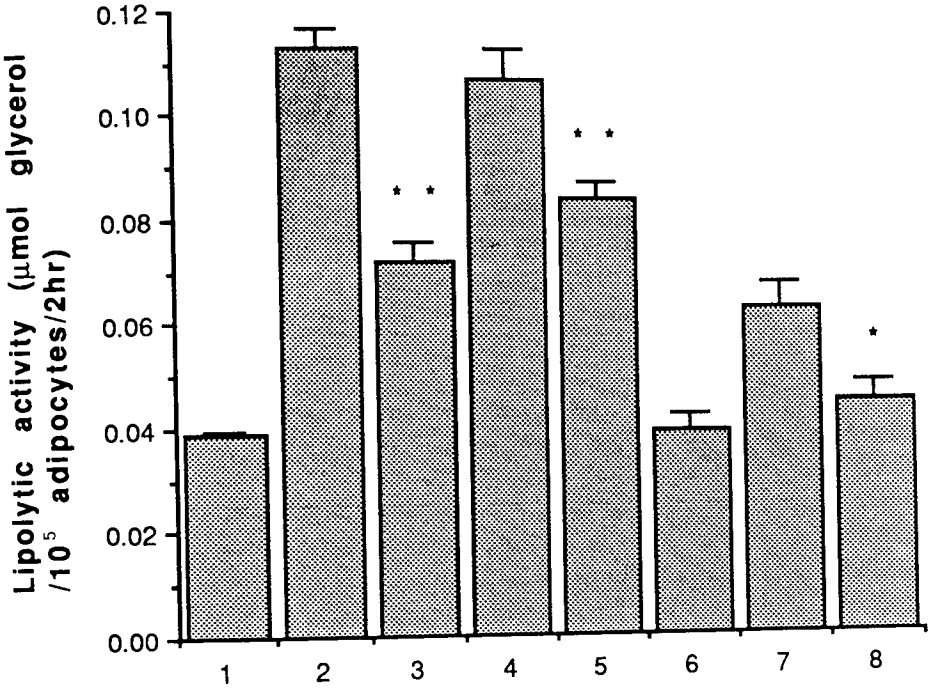
8 = fat cells + forskolin (25µM) + EPA (331µM)

Results are expressed as mean ± SEM of 4 experiments.

*p < 0.005, **p < 0.001 (compared with agonist, STT).

Figure 4.40(b)

Effect of DHA on Lipolytic Activity Produced by Isoprenaline, Dibutyryl cAMP and Forskolin in Mouse Adipocytes



1 = fat cells

2 = fat cells + isoprenaline (1µM)

3 = fat cells + isoprenaline (1µM) + DHA (331µM)

4 = fat cells + dibutyryl cAMP (1mM)

5 = fat cells + dibutyryl cAMP (1mM) + DHA (331µM)

6 = fat cells + DMSO

7 = fat cells + forskolin (25µM)

8 = fat cells + forskolin (25µM) + DHA (331µM)

Results are expressed as mean ± SEM of 4 experiments.

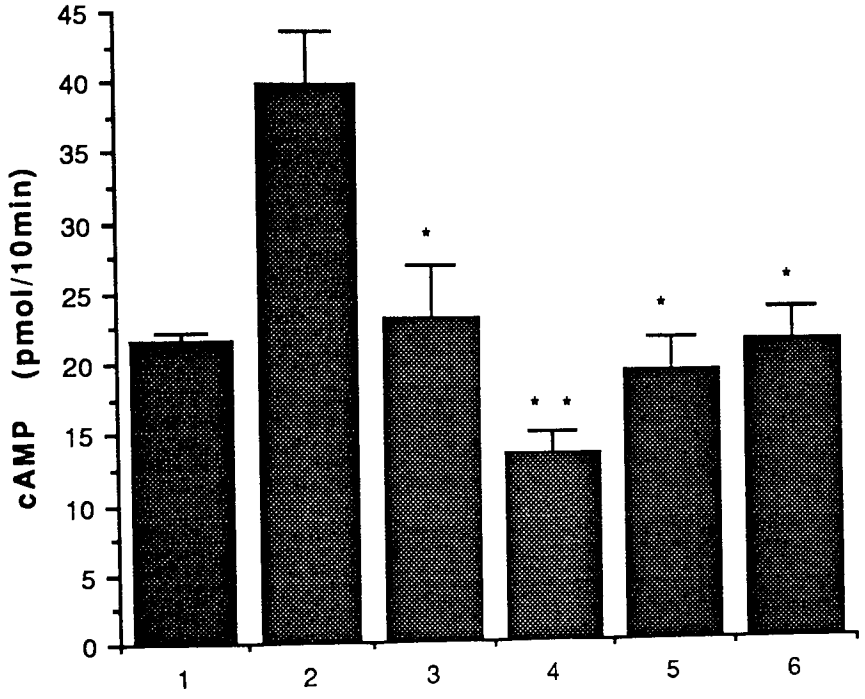
*$p < 0.05$, **$p < 0.005$ (compared with agonist, STT).

adenylate cyclase activity.

In summary, the polyunsaturated fatty acids, EPA, DHA and AA exert an inhibitory effect on adenylate cyclase activity which is reflected by their effect on lipolysis. There appear to be several components to this inhibition. Arachidonic acid, an ω-6 fatty acid, appeared to possess a slightly different inhibitory profile on adenylate cyclase and lipolytic activity to the ω-3 fatty acids, but the actions of EPA and DHA were indistinguishable except when the pH of the vehicle was altered. A difference in the actions of these two fatty acids was required in order to explain the lack of an anti-cachectic effect by DHA in animal studies (Tisdale and Beck, 1991). The difference observed with a change in pH may explain the difference in the actions of EPA and DHA in vivo. A change in the degree of ionization of the fatty acids may alter the extent of incorporation into the adipocyte plasma membrane which may, in turn, lead to the loss of one or more of the hypothesized components of the inhibitory action of these fatty acids. It should be noted that incorporation of fatty acids into membrane phospholipids may not have occurred in the in vitro situation, although this may be an important factor in vivo. The cell components necessary for the exchange of exogenous fatty acids with endogenous fatty acids within the plasma membrane may not be present in the membrane preparations utilized in this study. Therefore, although analysis of membrane EPA levels revealed increasing amounts of this fatty acid with increasing concentration of EPA in the

Figure 4.41

Effect of Changing pH of Vehicle for EPA and DHA



1 = basal

2 = isoprenaline (1μM)

3 = isoprenaline (1μM) + EPA (248μM)

4 = isoprenaline (1μM) + EPA (496μM)

5 = isoprenaline (1μM) + DHA (248μM)

6 = isoprenaline (1μM) + DHA (496μM)

Results are expressed as mean ± SEM of 3 - 4 experiments.
Membrane concentration = 50μg/100μl.
pH of vehicle changed from 9 (equimolar lysine) to 10 (50% lysine/50% Na_2CO_3).
*p < 0.05, **p< 0.005 (compared with iso, STT).

incubation medium, the results obtained may indicate insertion of the fatty acid into the membrane without incorporation into membrane phospholipids. The inhibitory effect of the fatty acids, which occurred at relatively high fatty acid : plasma membrane ratios, may have been mediated, at least in part, by G_i but did not appear to be linked to receptor activation of this G-protein. A direct interaction of the fatty acids with the catalytic unit of adenylate cyclase has also been proposed as a component of the inhibitory mechanism. The stimulatory effect observed with the same fatty acids occurred with higher fatty acid : plasma membrane concentrations and may have involved G_s. The effects of fatty acids on cAMP production appeared to correlate with their effects on lipolysis. Only the inhibitory effect was observed when studying lipolytic activity which could be explained by the fact that the number of isolated cells in each sample was equivalent to < 50µg membrane protein.

A final point which must not be overlooked is the possibility of a detergent effect of the lysine salt of the fatty acids on the adipocyte plasma membrane. If the critical micelle concentration had been exceeded, the effects observed may have been due to disruption of the plasma membrane and solubilization of membrane components. This effect would have been sensitive to pH changes. The effect of increasing concentrations of a known detergent on adenylate cyclase activity should therefore be investigated.

4.3.1.3 Investigation of the Interaction of Eicosapentaenoic Acid with the Phosphoinositide System

4.3.1.3.1 Introduction

The process by which adenylate cyclase responds to hormonal stimulation is now well known. A signal transduction pathway which, until recently, was relatively unknown is that which regulates the phosphoinositide system. Hokin and Hokin (1955) first suggested the role of phosphoinositide metabolism in receptor-mediated intracellular events. The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C in the plasma membrane generates two second messenger molecules; diacylglycerol (DAG) which activates protein kinase C in the plasma membrane, and inositol 1,4,5-triphosphate (IP_3) which is released into the cytoplasm and mobilizes calcium stores from the endoplasmic reticulum (Berridge and Irvine, 1984). Whereas adenylate cyclase is known to be stimulated and inhibited by specific G-proteins, namely G_s and G_i respectively, the G-protein pathway responsible for stimulating phospholipase C has yet to be fully characterized. Many receptors are known to stimulate this pathway (Fain, 1990) but it is not yet known whether receptors may inhibit its activity. Goodhardt *et al* (1982) were the first to indicate a potential link between guanine nucleotides and calcium-mobilizing receptors. This was followed by studies by Nakamura and Ui (1985) and Ohta *et al*

(1985) who discovered a 41 kDa pertussis toxin substrate which was proposed to mediate receptor-linked activation of phospholipase C activity. Discoveries such as these led to the premature conclusion that the α-subunit of G_i, which regulates the adenylate cyclase signal transduction pathway, was also the sole G-protein responsible for the activation of phospholipase C. However, Hepler and Harden (1986) and Martin et al (1986) discovered a GTP-dependent pathway stimulating phospholipase C which was unaltered by treatment with pertussis toxin. In addition to this, Oinuma et al (1987) purified a 40 kDa GTP-binding protein from differentiated HL60 cells which was a substrate for ADP-ribosylation catalysed by pertussis toxin but was discovered to be neither $G_i\alpha$ nor $G_o\alpha$. It is not yet known whether this polypeptide plays a role in calcium mobilization and phosphoinositide hydrolysis. Nevertheless, the guanine nucleotide-dependent interaction between calcium-mobilizing receptor and effector molecules is more complex than was originally thought.

The chemotactic peptide, formylmethionylleucyl-phenylalanine (f-Met-Leu-Phe), stimulated the formation of IP_3 in plasma membranes prepared from differentiated HL60 cells apparently via a pertussis toxin - sensitive 40 kDa G-protein α-subunit (Kikuchi et al, 1986). This polypeptide has been suggested to be $G_{i-2}\alpha$ (Murphy et al, 1987; Goldsmith et al, 1987) and, more recently, it has been suggested that $G_{i-3}\alpha$ may also interact with the f-Met-Leu-Phe receptor (Gierschik et al, 1989). The existence of this receptor which is coupled to

the phosphoinositide system in addition to interacting with a G-protein known to be involved in the hormonal inhibition of adenylate cyclase (in other cell types, although curiously not in HL60 cells (Gierschik et al, 1989)) provides an opportunity to investigate a link between these two signal transduction pathways.

Thompson et al (1988) described an experiment in which the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited the chemotactic peptide - induced elevation of intracellular calcium in differentiated HL60 cells. The ability of phorbol esters to reduce elevated calcium levels in addition to inhibiting phosphoinositide metabolism has also been reported by MacIntyre et al (1985) and Orellana et al (1985). This inhibitory effect of phorbol esters, which are known to activate protein kinase C (PKC), was attributed to a negative feedback mechanism mediated by this enzyme on phospholipase C activity. An interaction of unsaturated fatty acids with protein kinase C leading to stimulation of this enzyme has been suggested by Speizer et al (1991) and Asaoka et al (1992) and may indicate an intracellular modulatory role of these fatty acids. Protein kinase C itself has complex intracellular roles which include interactions with the adenylate cyclase system. Exactly how PKC affects adenylate cyclase is not known and reports to date are conflicting. Naghshineh et al (1986) suggest a stimulatory effect as a result of direct interaction of PKC with a component of the adenylate cyclase system within the adipocyte plasma

membrane. This finding supported those of Bell et al (1985) and Sugden et al (1985) who reported that phorbol esters increase adenylate cyclase activity. However, Sibley et al (1984) and Kelleher et al (1984) reported phosphorylation of β-adrenergic receptors and subsequent desensitization of adenylate cyclase as a result of treatment of cells with phorbol esters. In addition to this, Deaciuc and Spitzer (1991) reported that TPA exerted an insulin-like inhibitory effect on the hormonal stimulation of adipocyte lipolysis which is mediated by adenylate cyclase.

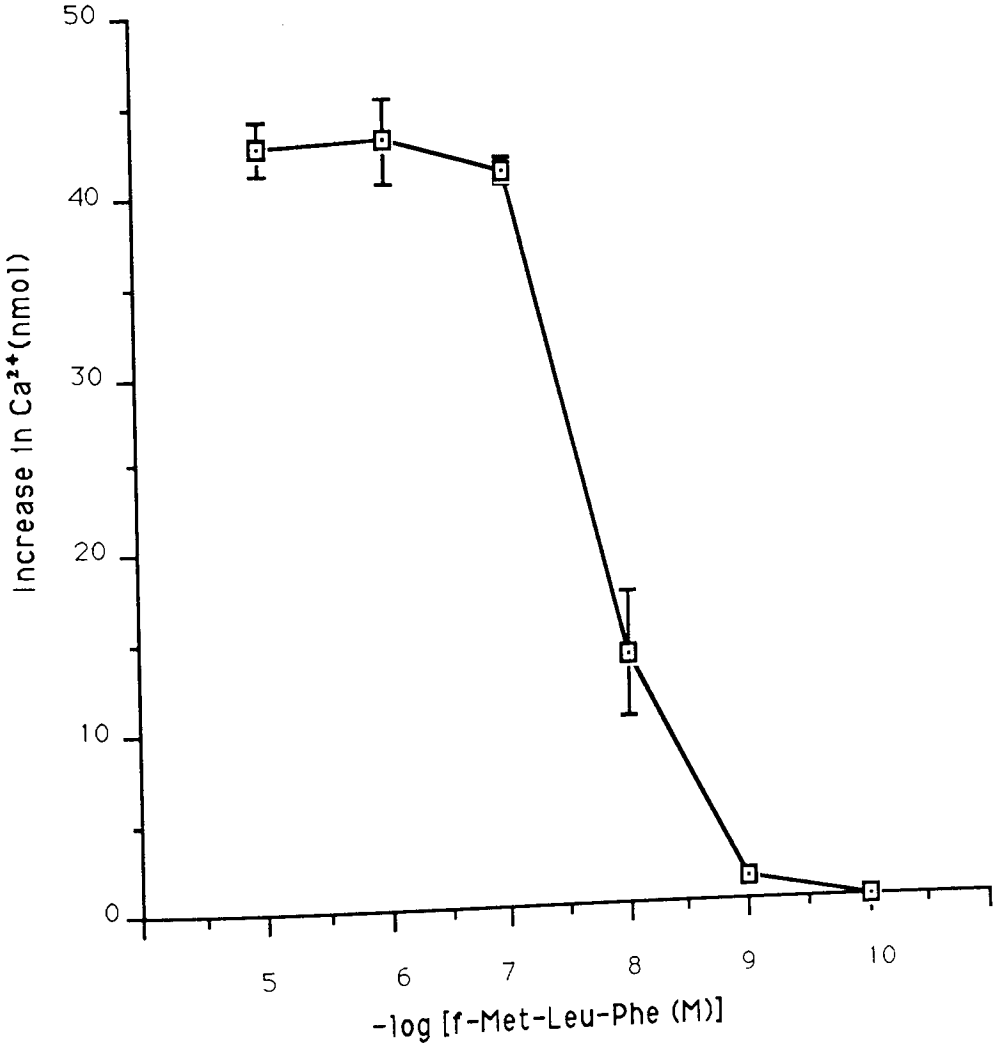
The object of this experiment was therefore to investigate the effect of eicospentaenoic acid on the intracellular calcium rise in differentiated HL60 cells induced by the chemotactic peptide f-Met-Leu-Phe and assess the consequences of such an interaction.

4.3.1.3.2 Results

Formyl-Met-Leu-Phe produced a concentration-dependent rise in intracellular calcium levels with maximum activity at a concentration of 10^{-6}M (fig. 4.42). HL60 cells treated with EPA at concentrations of 165μM and 331μM did not produce a rise in calcium levels and did not reduce the rise in intracellular calcium induced by f-Met-Leu-Phe (fig. 4.43). A time course for intracellular calcium rise after the addition of f-Met-Leu-Phe in EPA-treated cells showed no difference from controls over 2.5 min (figs. 4.44(a), (b) and (c)).

Figure 4.42

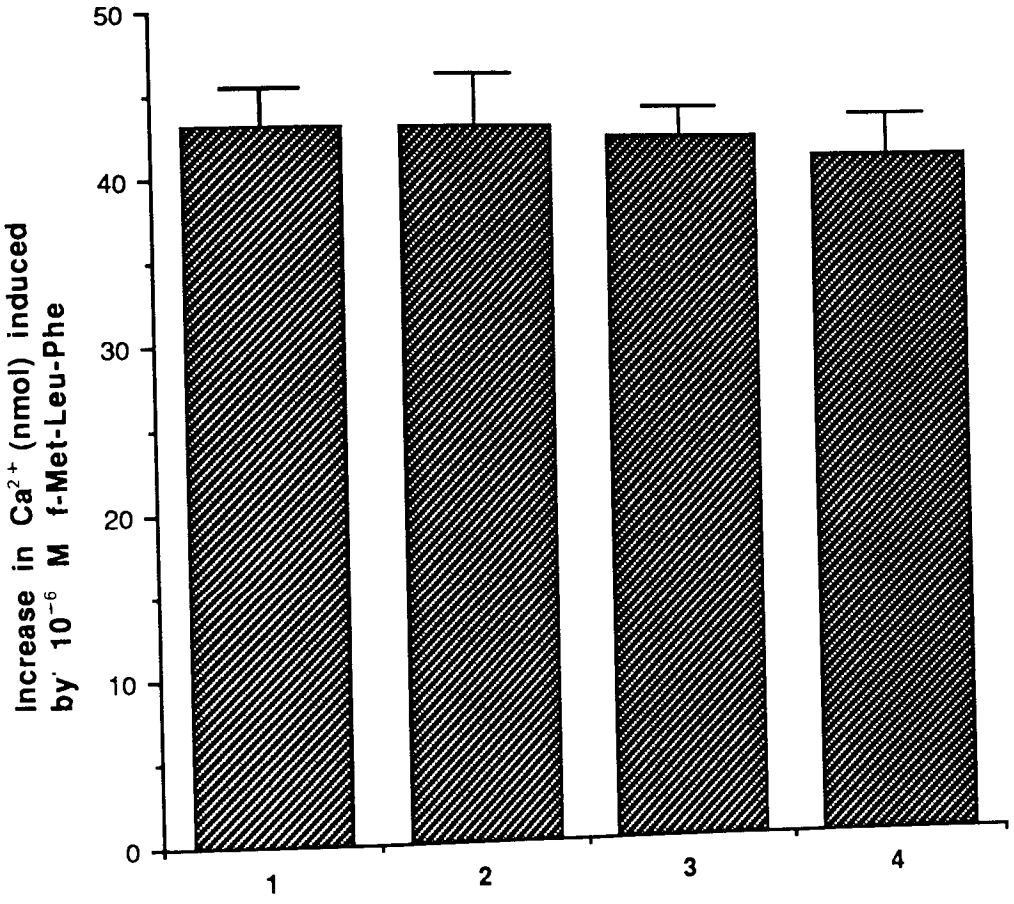
Dose-Response Relationship For f-Met-Leu-Phe



Results are expressed as mean ± SEM of 3 experiments.

Figure 4.43

Effect of Eicosapentaenoic Acid on Maximum Intracellular Calcium Rise Induced By f-Met-Leu-Phe



1 = f-Met-Leu-Phe (10^{-6}M)

2 = f-Met-Leu-Phe (10^{-6}M) + EPA (165μM)

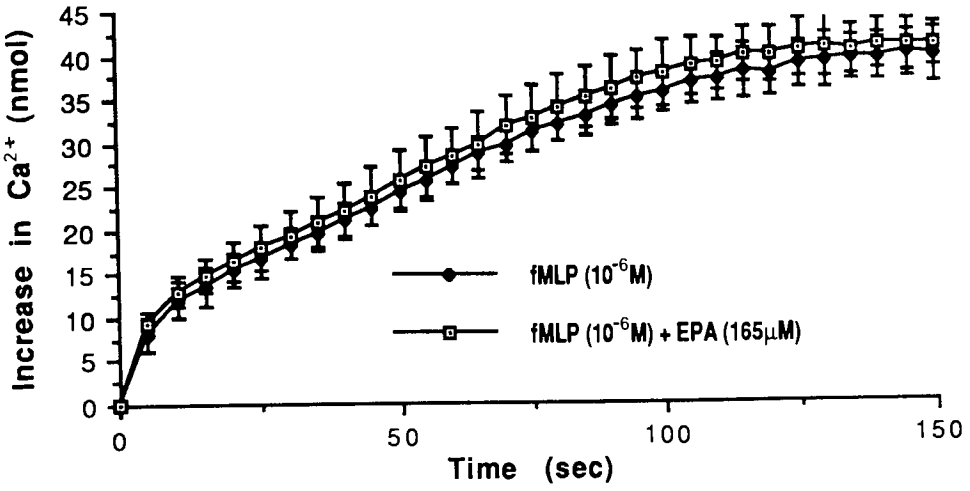
3 = f-Met-Leu-Phe (10^{-6}M) + EPA (331μM)

4 = f-Met-Leu-Phe (10^{-6}M) + EtOH

Results are expressed as mean ± SEM of 3 - 5 experiments.

Figure 4.44(a)

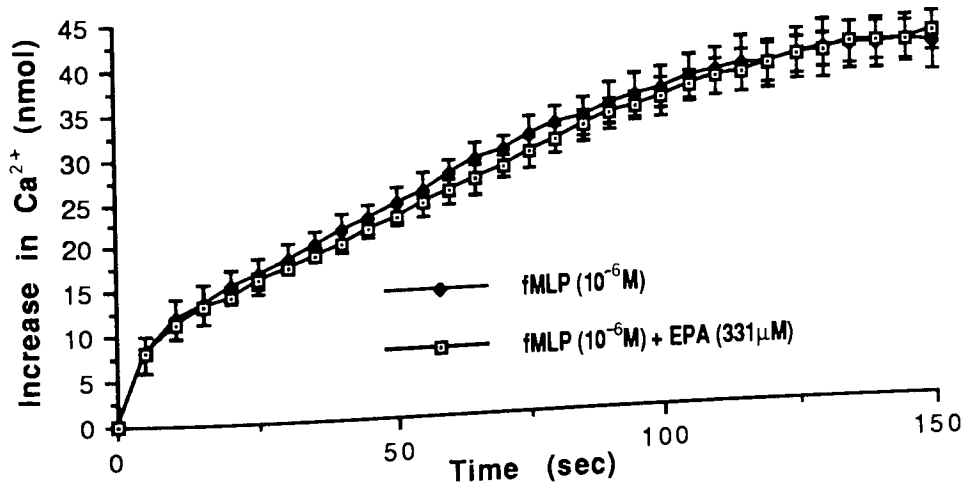
Effect of Eicosapentaenoic Acid (165μM) on the Time Course of f-Met-Leu-Phe - Induced Intracellular Calcium Rise



Results are expressed as mean ± SEM of 3 experiments.

Figure 4.44(b)

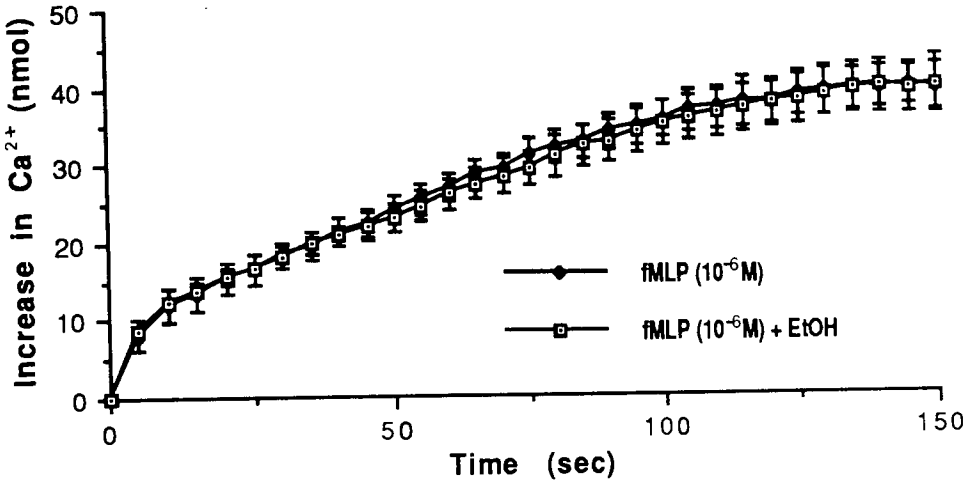
Effect of Eicosapentaenoic Acid (331μM) on the Time Course of f-Met-Leu-Phe - Induced Intracellular Calcium Rise



Results are expressed as mean ± SEM of 3 experiments.

Figure 4.44(c)

Effect of Ethanol on the Time Course of f-Met-Leu-Phe - Induced Intracellular Calcium Rise



Results are expressed as mean ± SEM of 3 experiments.

4.3.1.3.3 Discussion

The inability of EPA to affect the intracellular calcium level, either in the resting state of HL60 cells or on stimulation with f-Met-Leu-Phe, would seem to indicate that EPA does not interact with G_i in this system. One explanation for this effect may be that EPA acts through a specific membrane receptor which does not exist in HL60 cells. Studies by Tisdale and Beck (1991), however, indicate that EPA exerts an inhibitory effect on the lipolysis produced by a wide range

of stimuli in adipocytes, which would appear to indicate a less specific interaction of this fatty acid with cell components. The inhibition of f-Met-Leu-Phe - induced intracellular calcium rise observed by Thompson et al (1988), which was attributed to negative feedback regulation of phospholipase C by protein kinase C, was not observed in this experiment. This finding suggests that EPA does not interact with PKC to a sufficient extent to cause its activation in this assay system. The apparent absence of an effect of EPA on PKC disagrees with the stimulation of PKC observed by Speizer et al (1991) and Asaoka et al (1992) with various unsaturated fatty acids (including EPA), although caution must be exercised when comparing these findings with the results of this experiment due to the fact that the studies quoted were performed on crude preparations of PKC and not in whole cells.

It would be unwise at this stage to completely eliminate the possibility of an interaction of EPA with the components of the phospholipase C/protein kinase C system due to the complexities and relative lack of understanding of its operation and physiological significance compared with the well-characterized adenylate cyclase system. There are also factors which complicate the transfer of information from the situation in HL60 cells to adipocytes. For instance, $G_{i-2}\alpha$, which is known to mediate the inhibition of adenylate cyclase in more than one cell type (Simonds et al, 1989; McKenzie et al, 1990) has been shown to interact with the f-Met-Leu-Phe receptor and subsequently stimulate phospholipase C in HL60

cells (Gierschik et al, 1989), yet this receptor is not functionally coupled to adenylate cyclase in this cell type. This indicates that the ability of a G-protein α-subunit to activate or inhibit a particular pathway may not be determined by its primary structure alone. Its function may be dependent on additional factors such as compartmentalization and relative expression of the G-protein in a particular tissue compared with other membrane components and the existence of distinct subtypes of effector molecules in different cell types.

Thus, the only conclusion that can positively be drawn from this experiment is that EPA does not interact with the components of the f-Met-Leu-Phe receptor/phospholipase C/protein kinase C system in HL60 cells. In order to determine the specificity of the interaction of EPA (and other fatty acids) with G_i subtypes and/or associated receptors, further studies in other cell types and more detailed receptor-binding studies are required.

4.3.2 Investigation of BW A4C as an Inhibitor of Cancer Cachexia

4.3.2.1 Introduction

Arachidonic acid is a fundamental constituent of cells and is the principal eicosanoid precursor. It may be converted to prostaglandins and thromboxanes via the cyclooxygenase pathway or to leukotrienes and hydroxy fatty acids via the lipoxygenase pathway. Following the implication of the role of prostaglandins in tumour growth, recent research has implicated the involvement of the lipoxygenase products of arachidonic acid in cancer. Linoleic acid, an essential fatty acid which has been associated with enhanced tumour growth in animal models (Ip, 1987; Wicha et al, 1979), can be converted to arachidonic acid in many tissues (Buckman et al, 1991). This, and the observation by Sauer and Dauchy (1988) that linoleic and arachidonic acids supported tumour growth, suggested a role for arachidonic acid metabolites in tumour growth. Buckman et al (1991) suggested that the products of lipoxygenase rather than cyclooxygenase played a major role in linoleate-stimulated tumour growth. They noted that although linoleate was converted to prostaglandins E_1 and E_2, these prostaglandins did not stimulate the growth of 4526 mouse mammary tumour cells in vitro. In addition to this, cyclooxygenase inhibitors, which inhibited prostaglandin production, stimulated tumour growth whilst inhibitors of 5-

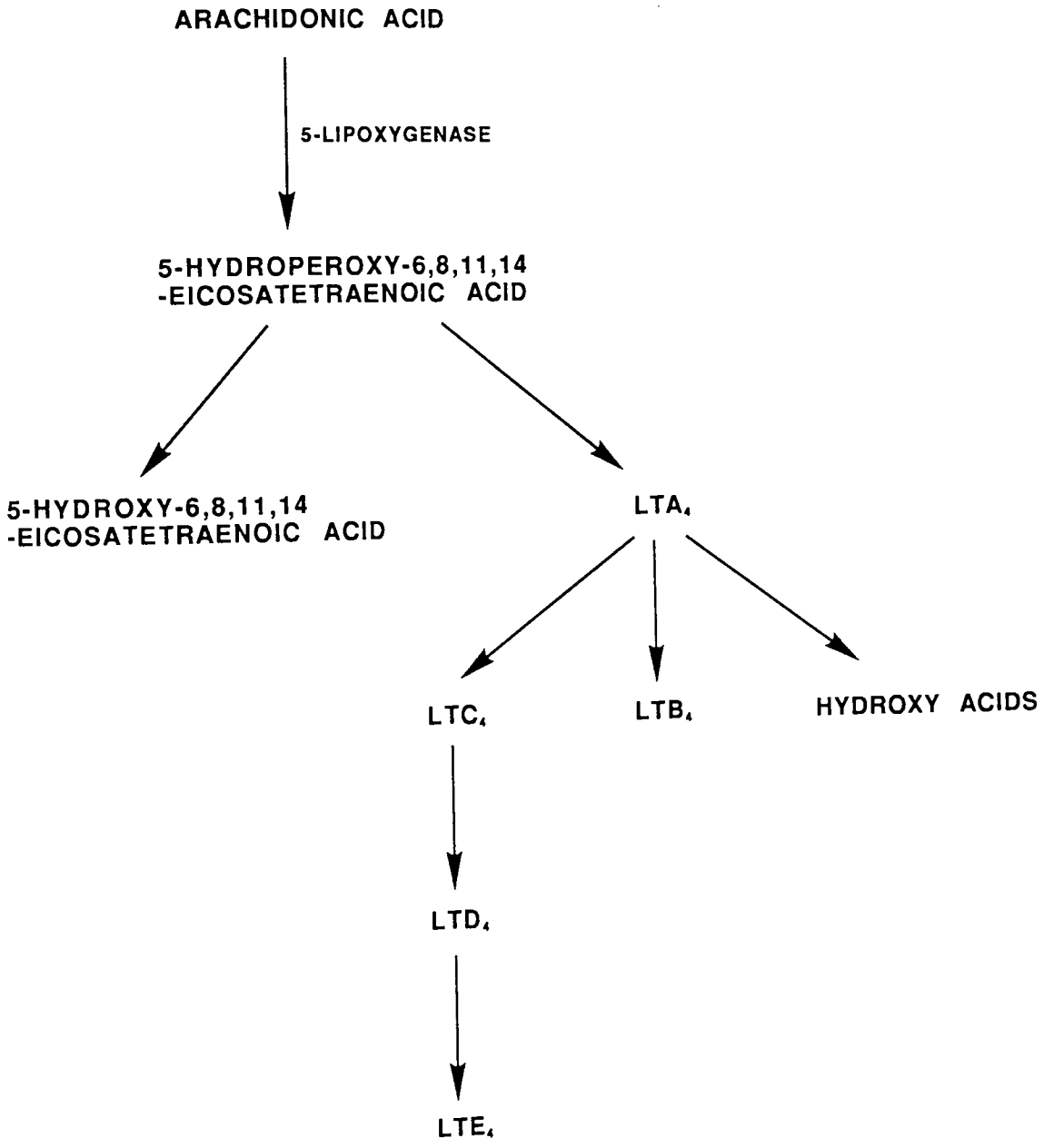
lipoxygenase decreased growth. Glasgow and Eling (1990) have suggested the involvement of hydroxy fatty acid derivatives from the lipoxygenase metabolism of exogenous linoleate in fibroblast mitogenesis. Further evidence for the involvement of lipoxygenase products in tumour growth is given by the observation that oleate, which is not metabolized to prostaglandins but is a substrate for lipoxygenase (Willis, 1987), enhances tumour growth in vivo (Buckman et al, 1990) and in vitro (Wicha et al , 1979).

Bandyopahyay et al (1988) suggested a synergistic relationship between 5-, 12- and 15-HETEs (lipoxygenase metabolites) and PGE_2 (cyclooxygenase metabolite). Of the three lipoxygenase products, 5-HETE was retained intracellularly to the greatest extent which supports the suggestion that this metabolite may be the important factor affecting cell growth. Products of the 5-lipoxygenase pathway are shown in fig. 4.45.

The above discoveries led to an investigation of the anti-tumour effect of a selective 5-lipoxygenase inhibitor, N-(3-phenoxycinnamyl)acetohydroxamic acid (BW A4C) (fig. 4.46). This investigation (Beck, S.A. and Hudson, E.A., unpublished results) showed that BW A4C did exhibit an inhibitory effect on MAC16 tumour growth with an accompanying reduction in the cachexia produced by this tumour.

The object of this study was to investigate whether the mechanism effecting the reversal of cachexia was distinct from the anti-tumour effect as it appears to be with the ω-3

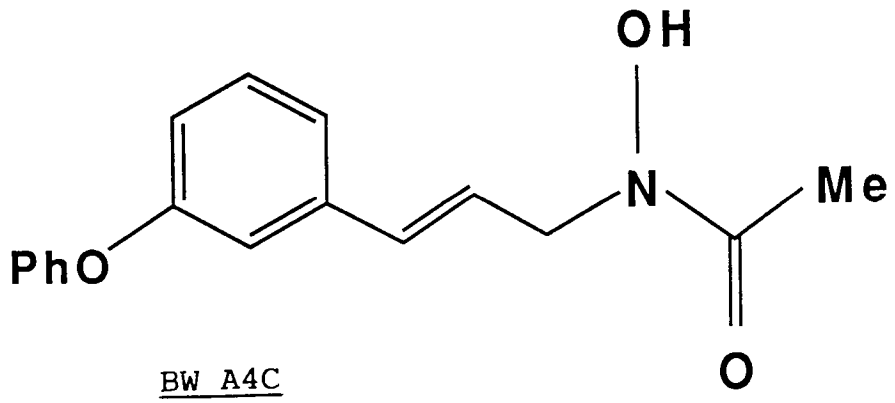
Figure 4.45



Products of the 5-Lipoxygenase Pathway

polyunsaturated fatty acid, eicosapentaenoic acid. The effect of BW A4C on adipocyte lipolysis in vitro was investigated with a range of agonists. Indomethacin, a cyclooxygenase inhibitor, which has been shown to inhibit tumour growth (Narisawa et al, 1981) and produces a small reversal of the cachectic state with a reduction in growth of the MAC16 tumour (Beck, S.A. and Hudson, E.A., unpublished results) was used for comparison of the anti-lipolytic property of BW A4C.

Figure 4.46



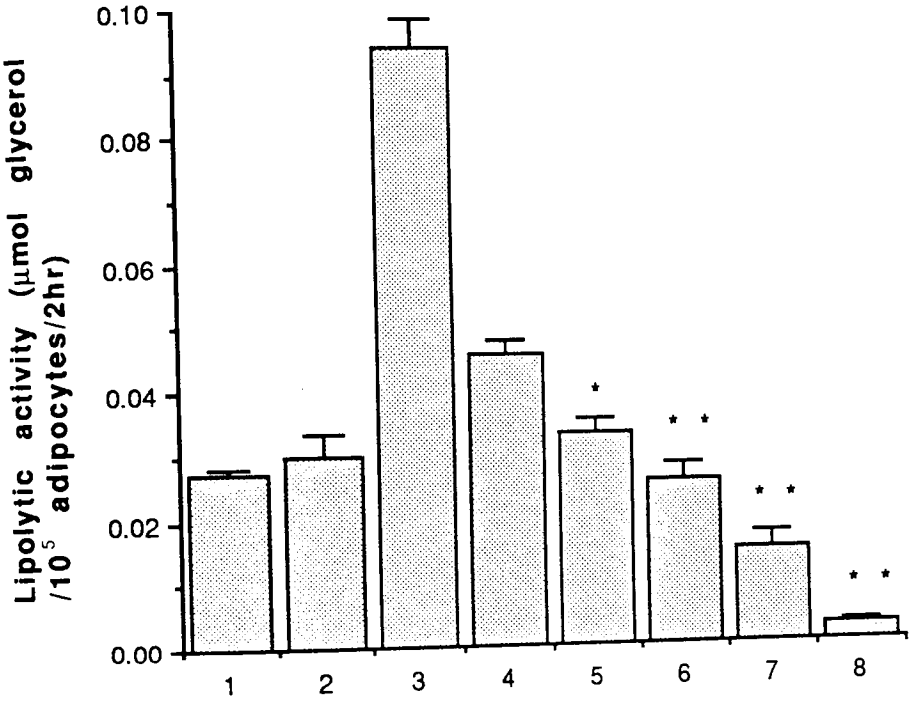
BW A4C

4.3.2.2 Results

BW A4C produced a significant dose-related inhibition of lipolysis in isolated mouse adipocytes stimulated by isoprenaline (fig. 4.47), ACTH (fig. 4.48) and MAC16 tumour homogenate (fig. 4.49) in addition to significantly reducing basal lipolysis (fig. 4.50). Indomethacin also significantly inhibited isoprenaline-stimulated lipolysis in a dose-related

Figure 4.47

Effect of BW A4C on Isoprenaline-Stimulated Lipolysis in Mouse Adipocytes



1 = fat cells

2 = fat cells + DMSO

3 = fat cells + isoprenaline (1µM)

4 = fat cells + isoprenaline (1µM) + DMSO

5 = fat cells + isoprenaline (1µM) + BW A4C (177µM)

6 = fat cells + isoprenaline (1µM) + BW A4C (353µM)

7 = fat cells + isoprenaline (1µM) + BW A4C (883µM)

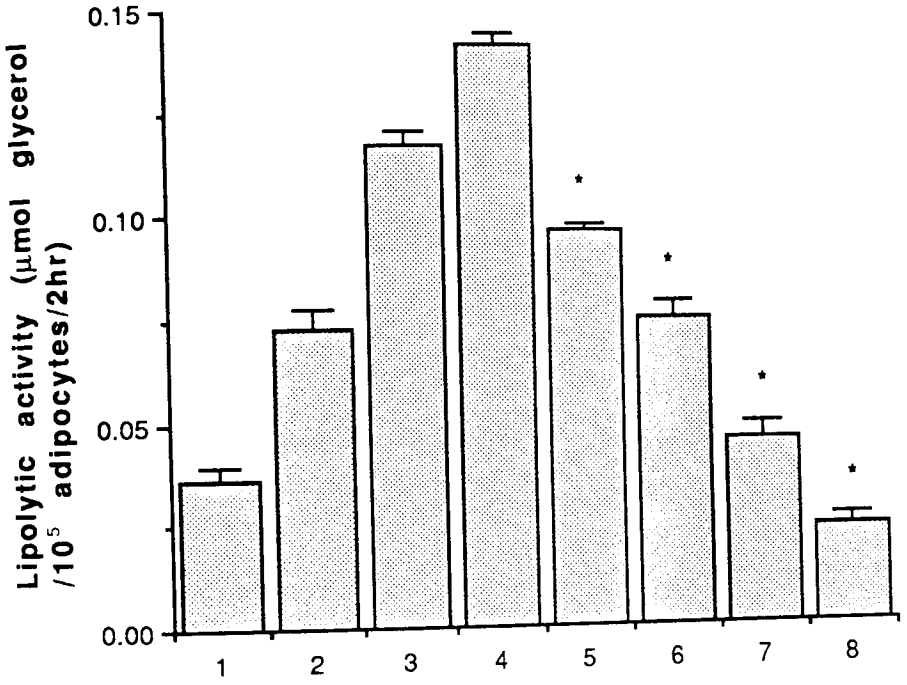
8 = fat cells + isoprenaline (1µM) + BW A4C (1767µM)

Results are expressed as mean ± SEM of 4 experiments.

*p < 0.005, **p < 0.001 (compared with iso + DMSO, STT).

Figure 4.48

Effect of BW A4C on ACTH - Stimulated Lipolysis in Mouse Adipocytes



1 = fat cells

2 = fat cells + DMSO

3 = fat cells + ACTH (25U)

4 = fat cells + ACTH (25U) + DMSO

5 = fat cells + ACTH (25U) + BW A4C (177µM)

6 = fat cells + ACTH (25U) + BW A4C (353µM)

7 = fat cells + ACTH (25U) + BW A4C (883µM)

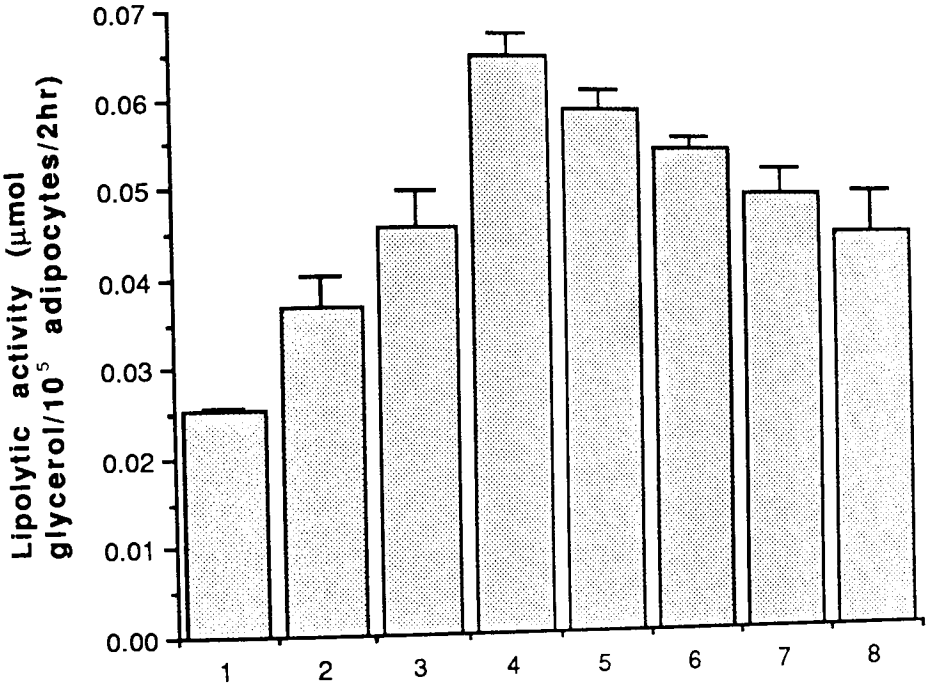
8 = fat cells + ACTH (25U) + BW A4C (1767µM)

Results are expressed as mean ± SEM of 4 experiments.

*p < 0.001 (compared with ACTH + DMSO, STT).

Figure 4.49

Effect of BW A4C on MAC16 Lipolytic Factor - Stimulated Lipolysis in Mouse Adipocytes



1 = fat cells

2 = fat cells + DMSO

3 = fat cells + MAC16 tumour homogenate (100µl)

4 = fat cells + MAC16 tumour homogenate (100µl) + DMSO

5 = fat cells + MAC16 tumour homogenate + BW A4C (177µM)

6 = fat cells + MAC16 tumour homogenate + BW A4C (353µM)

7 = fat cells + MAC16 tumour homogenate + BW A4C (883µM)

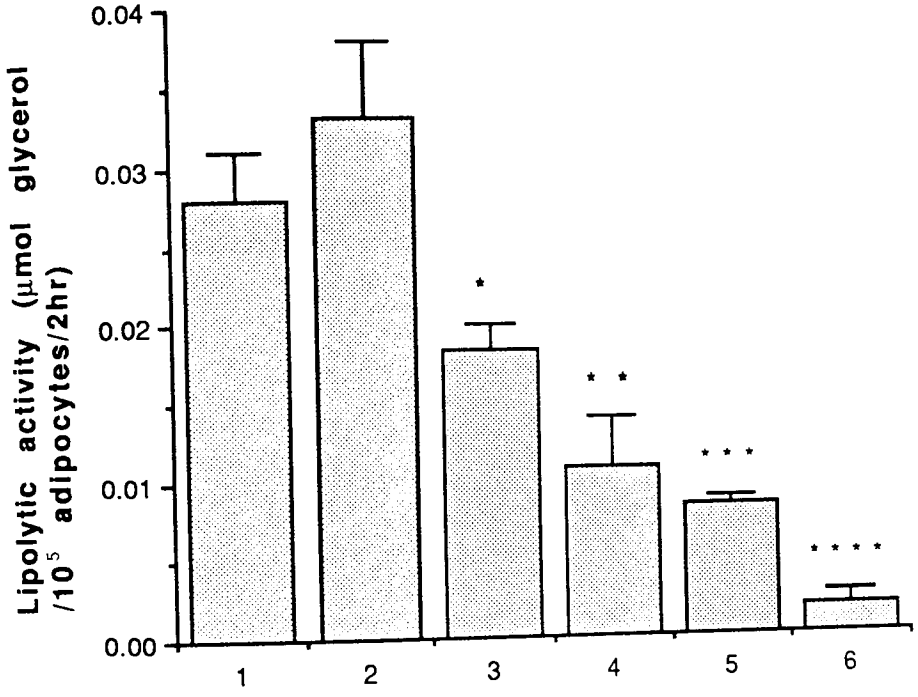
8 = fat cells + MAC16 tumour homogenate + BW A4C (1767µM)

Results are expressed as mean ± SEM of 4 experiments.

*p < 0.05, **p < 0.01, ***p < 0.001 (compared with factor + DMSO, STT).

Figure 4.50

Effect of BW A4C on Basal Lipolysis in Mouse Adipocytes



1 = fat cells

2 = fat cells + DMSO

3 = fat cells + BW A4C (177µM)

4 = fat cells + BW A4C (353µM)

5 = fat cells + BW A4C (883µM)

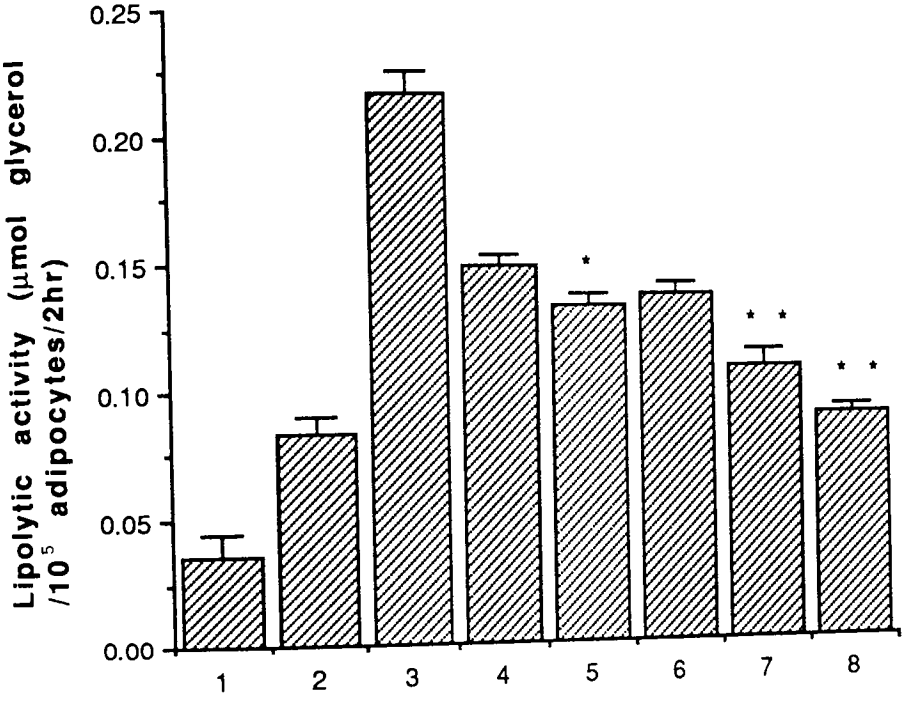
6 = fat cells + BW A4C (1767µM)

Results are expressed as mean ± SEM of 4 experiments.

*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001 (compared with fat cells + DMSO, STT).

Figure 4.51

Effect of Indomethacin on Isoprenaline-Stimulated Lipolysis in Mouse Adipocytes



1 = fat cells

2 = fat cells + DMSO

3 = fat cells + isoprenaline (1µM)

4 = fat cells + isoprenaline (1µM) + DMSO

5 = fat cells + isoprenaline (1µM) + indomethacin (140µM)

6 = fat cells + isoprenaline (1µM) + indomethacin (279µM)

7 = fat cells + isoprenaline (1µM) + indomethacin (699µM)

8 = fat cells + isoprenaline (1µM) + indomethacin (1397µM)

Results are expressed as mean ± SEM of 4 experiments.

*$p < 0.05$, **$p < 0.001$ (compared with iso + DMSO, STT).

manner (fig. 4.51) but this inhibition occurred at higher concentrations than a comparable inhibition due to BW A4C.

4.3.2.3 Discussion

The inhibition of lipolysis observed with BW A4C indicated a direct action on the adipocyte *in vitro*. If this mechanism also occurs *in vivo*, it could suggest an anti-cachectic action of this drug. More detailed studies of the mechanism of the anti-lipolytic action of BW A4C are required in order to determine exactly how it exerts this effect. Indomethacin also appears to exert a direct inhibitory effect on the isolated adipocyte and this may suggest a small anti-cachectic effect independent of the anti-tumour activity observed with this drug. However, the extent of the reversal of cachexia is unlikely to encourage further research in this area.

An important factor which must not be overlooked before expanding this preliminary investigation into the anti-lipolytic effect of BW A4C and indomethacin is that the doses used to inhibit lipolysis are much greater than the small micromolar concentrations required to inhibit 5-lipoxygenase and cyclooxygenase respectively (Tateson *et al*, 1988; Buckman *et al*, 1991). The possibility that the relatively high concentrations of these agents could have caused a decrease in adipocyte lipolysis by reducing cell viability has not yet been eliminated and therefore must be

investigated.

One disadvantage of the 5-lipoxygenase inhibitor, BW A4C, is that it has a short plasma half-life (Tateson et al, 1988) and therefore clinical success at this stage would be doubtful. However if this can be overcome during formulation of the drug for use in clinical practice and if similar, but longer-acting compounds, also display anti-lipolytic activity, further research into these 5-lipoxygenase inhibitors may provide a relatively non-toxic form of chemotherapy with an additional distinct anti-cachectic effect.

SECTION 5 : CONCLUSION

The results from Section 4.1 further characterize the lipolytic factor produced by the MAC16 murine colon adenocarcinoma, which was studied by Beck and Tisdale (1987) because of the profound cachexia induced by this tumour in the absence of anorexia. This factor appears not to be an endogenous substance released in response to the presence of a tumour, but to be produced directly by the tumour cells. The MAC16 lipolytic factor produces lipolysis in isolated murine adipocytes (Beck and Tisdale, 1987) and increases cAMP production in murine adipocyte plasma membrane preparations (Section 4.3.1.2.1).

Curiously, the lipolytic factor does not produce lipolysis in isolated rat adipocytes (McDevitt, T., unpublished results). This reason for this recent observation is not at present understood, but may possibly be due to the lack of a receptor for the lipolytic factor in the rat adipocyte. In hindsight, the murine adipocyte would have been a better model for the present studies. The rat adipocyte was chosen because of its relatively large yield of adipose tissue per animal and was an acceptable model for the study of the inhibitory effect of EPA on isoprenaline-stimulated lipolysis and cAMP production. However, it should also be noted that the inhibitory effect of EPA and DHA on isoprenaline-stimulated lipolysis in mouse adipocytes appeared to be greater than that in rat adipocytes in this investigation. This has recently been confirmed by performing simultaneous, identical experiments on each preparation (Khan, S., unpublished

results). In addition to this observation, EPA (331μM) was found to inhibit adenylate cyclase activity stimulated by the MAC16 lipolytic factor at a membrane concentration of 100μg/100μl using mouse adipocyte plasma membranes, but enhanced isoprenaline-stimulated activity at this membrane concentration in the rat membrane preparation. The reason for the difference between these two species as regards the effect of MAC16 lipolytic factor and ω-3 fatty acids on adenylate cyclase and lipolytic activity in the adipocyte is at present unknown but may possibly be due to interspecies differences in the expression and compartmentalization of components of the adenylate cyclase pathway within the adipocyte plasma membrane. In the light of these observations, any further studies into the action of an agent which inhibits the cachexia due to the MAC16 tumour should be performed on the murine adipocyte. Further studies with purified lipolytic factor should produce a more detailed picture of its mechanism of action on the adipocyte.

The cachectic response to the MAC16 tumour, in addition to the growth of this tumour, is selectively reduced by the ω-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA) which is found in fish oil (Tisdale and Beck, 1991). This anti-cachectic agent was also found to inhibit lipolysis in isolated adipocytes with a range of lipolytic stimuli including the MAC16 lipolytic factor, and to reduce the elevation in cAMP levels observed due to this factor in isolated adipocytes (Tisdale and Beck, 1991). The other main

constituent of fish oil, docosahexaenoic acid (DHA), also an ω-3 polyunsaturated fatty acid, does not exhibit anti-tumour or anti-cachectic activity (Tisdale and Beck, 1991) and an investigation into the effect of EPA, DHA and gamma linolenic acid (GLA), a precursor for 1-series prostaglandins, on PGE_2 production by the spleen of animals bearing the MAC16 tumour, suggested that the anti-tumour effect of EPA did not involve the inhibition of prostaglandin production (Section 4.3.1.1). The anti-cachectic effect of EPA was greater than its anti-tumour effect (Beck et al, 1991) and hence the mechanism of this effect has been studied in great detail (Section 4.3.1.2).

Using isoprenaline as a model for lipolysis and cAMP production in the rat adipocyte, EPA, DHA and arachidonic acid (AA) were found to inhibit cAMP production with high ratios of fatty acid : plasma membrane and stimulate cAMP production with low ratios of fatty acid : plasma membrane. The exact mechanism of the observed alterations in adenylate cyclase activity due to these fatty acids is not known but may involve several components of the cascade system within the plasma membrane. The alterations in adenylate cyclase activity due to EPA, DHA and AA are dependent on the fatty acid : plasma membrane ratio and modified by the agonist : plasma membrane ratio (Section 4.3.1.2.3). There appears to be a GTP-dependent component of the inhibitory effect of EPA and DHA on adenylate cyclase activity although this does not seem to conform to the specifications for hormonal inhibition and may therefore

represent a direct interaction of the fatty acids with G_i. An interaction of EPA, DHA and AA with G_i, the inhibitory G-protein of the adenylate cyclase system, was shown in Section 4.3.1.2.2 and these results, showing the effect of the fatty acids on pertussis toxin - catalysed ADP-ribosylation, reflect the stimulatory/inhibitory pattern observed in the adenylate cyclase studies. The consequence of this interaction is not yet known but the inhibition of pertussis toxin - catalysed ADP-ribosylation of G_i by EPA in high concentrations may suggest that this G-protein is activated by EPA and that this interaction provides at least one component of the inhibitory mechanism of EPA on adenylate cyclase activity. EPA and DHA both inhibited lipolysis in isolated rat adipocytes in a dose-dependent manner, whereas inhibition due to AA was minimal and not dose-related. AA also differed from EPA and DHA in its action on adenylate cyclase in plasma membrane preparations. The actions of EPA and DHA on cAMP production and lipolysis were indistinguishable except when the pH of the vehicle used to dissolve the fatty acids was increased. This suggested that the degree of ionization may be an important factor in the search for an explanation for the differing actions of EPA and DHA on tumour growth and cachexia *in vivo*. Since EPA and DHA possess the ability to inhibit adenylate cyclase activity and lipolysis in identical concentration ranges *in vitro*, it seems likely that the reason for the lack of an anti-cachectic effect of DHA *in vivo* may be a difference in the extent of incorporation into adipocyte

plasma membranes. Lokesh et al (1988) noted that EPA was incorporated into phospholipids of mouse peritoneal macrophages to a greater extent than DHA. Philbrick et al (1987) noted significant uptake of EPA into all phospholipid fractions of cell membranes in the nervous tissue of rats fed a fish oil diet. Significant incorporation of DHA was only observed in the phosphoinositol fraction of the sciatic nerve. Croft et al (1987) observed that rats fed MaxEPA (EPA-rich diet) exhibited a significant decrease in plasma and tissue (liver and kidney) phospholipid arachidonic acid content with a large increase in EPA levels. Animals fed shark liver oil (rich in DHA) showed no alteration in liver and kidney phospholipid DHA levels and no decrease in arachidonic acid content, even though plasma levels of DHA increased on this diet. Nordøy et al (1986) noted that DHA was incorporated into total phospholipids of human endothelial cells when incubated with this fatty acid in vitro and this was associated with a reduction in arachidonic acid content. However, when cells were incubated with DHA and arachidonic acid together, the reduction in arachidonic acid was abolished and DHA uptake reduced. The ability of arachidonic acid to affect the incorporation of DHA into membrane phospholipids was also noted by Sinclair et al (1986). These researchers gave human subjects a diet of either southern Australian fish, rich in DHA and EPA or tropical Australian fish, rich in DHA and AA. Significant increases in the levels of the predominant fatty acids contained within these fish were noted in the plasma

lipids of subjects. However the platelet fatty acids levels reflected the fatty acid content of the fish only in the case of subjects fed the southern fish diet. Those fed the tropical fish diet exhibited increased levels of platelet AA but not of DHA. The above reports show differences in the extent of incorporation of the ω-3 fatty acids both *in vivo* and *in vitro*. These differences may be expected to alter the relevant physiological alterations attributed to the individual fatty acids. Although both EPA and DHA inhibited lipolysis and adenylate cyclase activity in the present investigation, the results of the research quoted above indicate that DHA may be poorly incorporated into cell membrane phospholipids *in vivo* and that one factor affecting the degree of incorporation of this fatty acid into cell membranes may be the level of ω-6 fatty acids present in the diet. Another factor affecting the incorporation of EPA and DHA into tissue lipid fractions may be the relative abundance of these fractions in a particular membrane. EPA and DHA are each preferentially incorporated into different classes of phospholipid in human erythrocyte membranes (Brown *et al*, 1991). It has been noted that EPA is readily incorporated into all phospholipid classes of human platelets *in vitro*. However, incorporation of this fatty acid *in vivo* is more selective, indicating specific control mechanisms which do not exist *in vitro* (von Schacky *et al*, 1985). Extending this explanation to the situation involving the incorporation of DHA into membrane phospholipids *in vivo*, the reason for the lack of an anti-cachectic effect in animals

treated with this fatty acid may be that the selectivity of incorporation of DHA *in vivo* is critical to its effect on adenylate cyclase activity. Hepatic metabolism of ω-3 fatty acids may also determine their incorporation into adipocyte plasma membranes. Zhang *et al* (1991) noted that DHA was a good substrate for triglyceride synthesis in the perfused rat liver. This was in contrast to EPA, which was found to be a poor substrate for triglyceride synthesis. Lin and Connor (1990) noted that DHA was deposited and retained in adipose triglyceride stores to a greater extent than EPA when rabbits were fed a fish oil - based diet. These results may indicate that DHA is preferentially incorporated into hepatic triglycerides which are secreted and stored in adipose tissue, and that EPA, being a poor substrate for triglyceride synthesis, is preferentially incorporated into the plasma membranes of adipocytes. Detailed studies of the incorporation of ω-3 and ω-6 polyunsaturated fatty acids into tissue lipid fractions are required in order to determine whether the lack of an anti-cachectic effect due to DHA is likely to be caused by poor incorporation of this fatty acid into adipocyte plasma membranes.

An investigation into the possibility of an interaction of EPA with other signal transduction pathways showed that this fatty acid did not alter f-Met-Leu-Phe - stimulated phospholipase C activity in HL60 cells. This result does not necessarily exclude the possibility of an interaction of EPA with the phosphoinositide system in other cell

types.

Finally, a preliminary investigation into the anti-cachectic effect of the 5-lipoxygenase inhibitor BW A4C, indicated that this compound may also exert a direct anti-lipolytic effect on the adipocyte. Further studies with this and similar compounds will determine whether they promise to be of use in cancer patients.

Eicosapentaenoic acid is to undergo clinical trials as an anti-tumour/anti-cachectic agent. Both this and the 5-lipoxygenase inhibitors produce a relatively low level of toxicity compared with conventional chemotherapeutic agents and therefore represent not only a novel method for the treatment of cancer and the associated cachectic process, but one which should produce minimal distress to the patient.

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APPENDIX

Publication

GROUDWATER, P., BECK, S.A., BARTON, C., ADAMSON, C., 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.

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Alteration of serum and urinary lipolytic activity with weight loss in cachectic cancer patients

P. Groundwater[1], S.A. Beck[1], C. Barton[2], C. Adamson[1], I.N. Ferrier[3] & M.J. Tisdale[1]

[1]*Cancer Research Campaign Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET;* [2]*CRC Clinical Trials Unit, Queen Elizabeth Hospital, Edgbaston, Birmingham B15 2TH; and* [3]*MRC Neurochemical Pathology Unit, Newcastle General Hospital, Newcastle upon Tyne NE4 6BE, UK.*