CATABOLIC FACTORS IN TUMOUR-INDUCED CACHEXIA

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Catabolic factors in tumour-induced cachexia

by

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A transplantable colon adenocarcinoma of the mouse (MAC16) was utilized as a model of human cancer cachexia. The MAC16 tumour produced extensive weight loss in the host at small tumour burdens and without a reduction in either food or fluid intake. The weight loss was characterised by a decrease in both carcass fat and muscle mass which were directly proportional to the weight of the tumour.

The weight loss has been correlated with the production of circulatory catabolic factors by the tumour, which degrade host muscle and adipose tissue in vitro.

These factors were further characterised and have been shown to be distinct and separable by gel exclusion chromatography. The proteolytic factors (molecular weight >150k daltons) were distinguishable from the lipolytic factors which appeared related with molecular weights of approximately 3.0, 1.5 and 0.7k daltons.

Lipolytic factors of the same molecular weights were identified in other tumour models and in the body fluids of tumour-bearing animals and cancer patients. These factors were not present in healthy individuals or in patients with other weight-losing conditions.

Various treatments studied reversed the weight loss seen in the cachexia induced by the MAC16 adenocarcinoma in vivo. The effects of these treatments could be linked in vitro to the inhibition of the catabolic factors produced by the tumour. These results suggest that these factors may be responsible for the cachexia the tumour confers on its host. These factors may be useful in the understanding and therapy of cancer cachexia.

Keywords: Cancer cachexia, weight loss, lipolytic factor, catabolic factors, MAC16 adenocarcinoma

TO MY PARENTS

AND

ANDREW

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Abbreviations

ACTH	adrenocorticotrophic hormone
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 5':3'monophosphate
DCH	docosahexaenoic acid
EDTA	ethylenediaminetetraacetic acid
EPA	eicosapentaenoic acid
FAA	flavoneacetic acid
FAB- mass spec	fast atom bombardment mass spectrometry
Glucose-6-P	glucose-6-phosphate
h	hour
hplc	high pressure liquid chromatography
3-HB	3-hydroxybutyrate
i.p.	intraperitoneal
MCT	medium chain triglycerides
Megace	megestrol acetate
min	minutes
n	number of experiments
NADH/NAD	nicotinamide adenine dinucleotide (reduced/oxidised)
p	probability
PG	prostaglandin
PMSF	phenylmethylsulphonyl fluoride
s	seconds
s.c.	subcutaneous
S.E.M.	standard error of the mean

TCA cycle	tricarboxylic acid cycle
TCA	trichloroacetic acid
TNF	tumour necrosis factor
TPN	total parenteral nutrition
tris	tris(hydroxymethyl)methylamine
UV	ultraviolet
wt	weight

All other abbreviations refer to SI units.

SECTION 1: INTRODUCTION

1.0 General Introduction

The word tumour was introduced by Galen around 164 A.D and comes from the greek word "tymbos" meaning a "sepulchural mound" and the latin word "tumere" meaning "to swell". The earliest references to what we now know as cancer are to be found before the time of Galen in the Ebers Papyrus which was written in Egypt in 1500 B.C (Braun 1977). Even now three and a half millenia later, many of the debilitating effects that tumours have on their hosts are poorly understood. All tumours are characterized by the fact that they grow at the expense of the host leading to extreme loss of weight and debilitation known as cachexia. Cachexia comes from the greek words kakos meaning "bad" and hexis meaning "condition" (Shamberger 1984).

1.1 Weight loss associated with cancer

The presence of cancer can produce significant nutritional deficiences in the tumour bearing host. Weight loss is an important diagnostic factor in patients with cancer and may influence the side effects of treatment, response of treatment and overall survival (Dewys et al 1980). The incidence of weight loss varies with the tumour type, stage of disease and performance status. Pancreatic and gastric cancer have the highest frequency of weight loss (83-87%) due to their site. While 48-61% of patients with colon, prostatic and lung cancers experience weight loss only 31-40% of patients with leukaemias, breast cancer and sarcoma lose weight at sometime during their illness. Another factor to be taken into account is the speed of

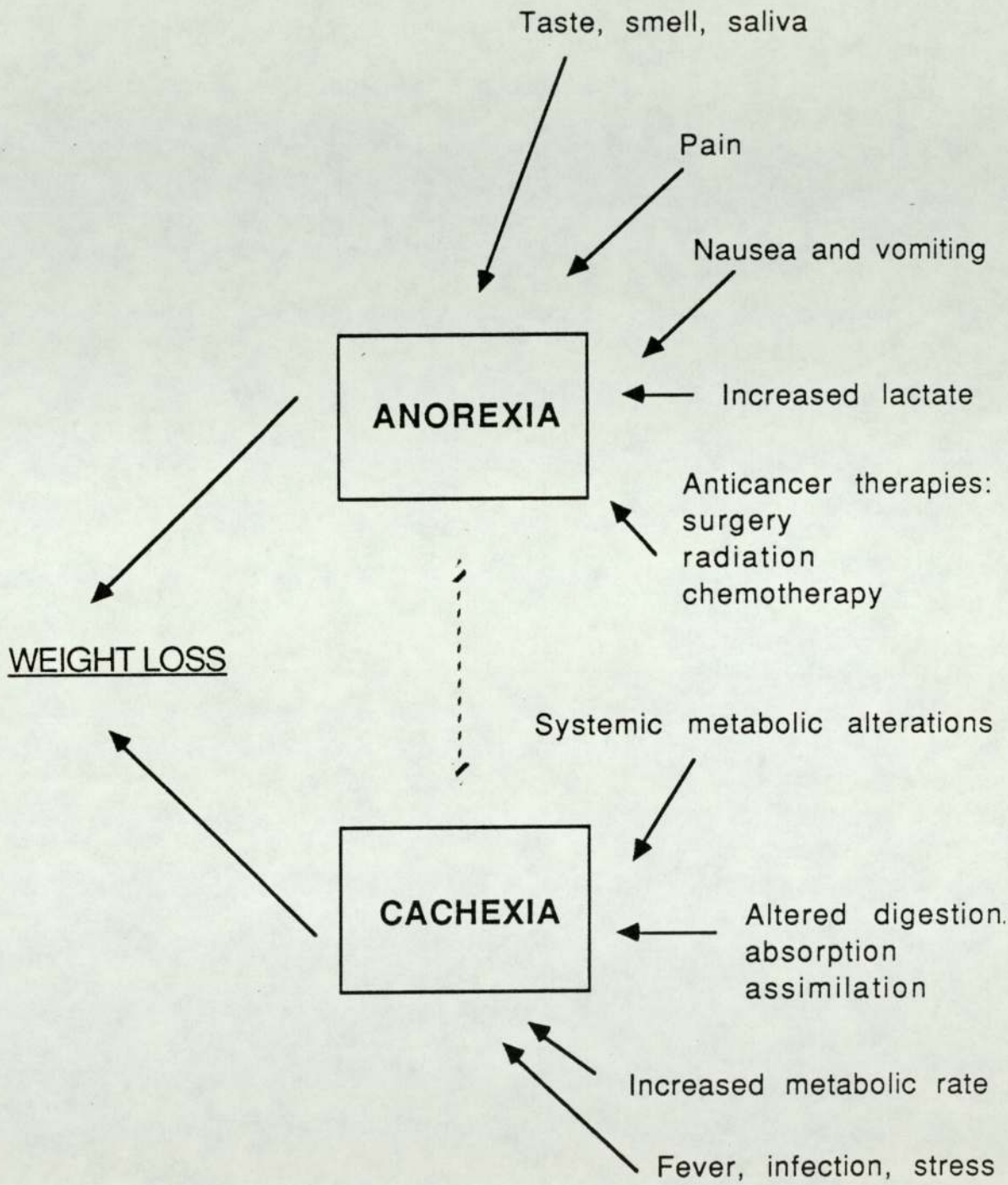
treatment. In patients with either operable or treatable tumours the patients are treated as quickly as possible before any weight loss can manifest itself. This is the case in breast cancer and sarcomas as the location of the tumour often leads to an early detection (Dewys 1986).

In addition to the disease itself the treatment given may have an effect on the nutritional status of the patient. Surgery is the first line method of treatment for almost all cancers of the alimentary tract. The amount of weight loss experienced after surgery depends on the site of the tumour and the effect the surgery has on the patients ability to swallow and take in food. The stress element of surgery will also cause a transient weight loss after the operation. Chemotherapy and radiation have side effects that result in the patient experiencing weight loss. Both chemotherapy and radiation can cause nausea and vomiting, stomatitis and an anorexic feeling (Kokal 1986). Thus this weight loss is a further complication in the treatment of cachexia in the cancer patient.

1.2 Cachexia/Anorexia syndrome

The relationship between cancer and nutrition is a complex one. Nutritional factors may influence the incidence and development of tumours. Once estabilished the cancer may cause nutritional abnormalities in the patient. Cancer cachexia has been recognised since 1932 (Warren, 1932) as a syndrome characterised by anorexia, weight loss, satiety, anaemia and asthenia (Lindsey 1986) (fig 1). Two thirds of cancer patients experience cachexia and it has

Figure 1



FACTORS INFLUENCING WEIGHT LOSS IN CANCER CACHEXIA

(Lindsey 1986)

been listed as the cause of death in many of these patients (Harnet 1952). Cancer patients may experience vast amounts of weight loss with no reduction in food intake. Therefore anorexia and cachexia may coexist, but cachexia may be experienced in patients with no anorexia who are eating normally or even larger amounts than usual.

1.3 Pathogenesis of Anorexia

Anorexia is the syndrome of loss of appetite, a loss of the desire to eat leading to a reduced food intake. Normally food intake is controlled by chemoreceptors located within the ingestive apparatus and by the physical effects of accumulation of ingested material in the intestinal tract. These chemoreceptors and volume receptors are regulated by neurotransmitters in the central nervous system (Dewys 1979).

The pathogenesis of anorexia is complex and cannot be attributed to any single factor. It has been proposed that sustained stimulation of receptors in the gastrointestinal tract results in satiety and decreased stimulation of appetite (Knox 1983). Theologides proposed that anorexia was due to the cancer itself. He hypothesised that the tumour secretes peptides, oligonucleotides and other small metabolites that produce anorexia via a peripheral effect on neuroendocrine cells and neuroreceptors and a direct effect on the hypothalamus. These tumour by-products may be mediated via the circulation. Increased lactate production has been postulated to be an anorexigenic factor (Baille et al 1970). Cachectin/TNF, a macrophage produced peptide, was

originally isolated as the mediator of wasting in chronic diseases by Rouzer and Cerami in 1980 (Cerami et al 1985). However recent reports suggest cachectin plays a major role in the pathophysiology of anorexia rather than cachexia (Mahony and Tisdale 1988, Stovroff et al 1988). No correlation between wasting and cachectin levels have been reported. However, no experiments have been designed to assess cachectin levels in anorexia in chronic diseases.

Changes in taste perception, food aversion and self-induced malnutrition are seen in some cancer patients leading to a decreased appetite. It has been suggested (Morrison 1976) that the immediate cause of weight loss seen in cancer cachexia is due to the failure of spontaneous food intake. In addition, the diagnosis of cancer alone may lead to a stress related anorexia. The location of the tumour can effect food intake, for instance head and neck cancers make it difficult to ingest, digest and absorb nutrients.

Weight loss in many patients is out of proportion to the decreased intake of nutrients and may even occur in the absence of anorexia.

1.4 Pathogenesis of Cachexia

1.4.1 Cancer cachexia

The characteristics of cachexia are:

1. anorexia and nausea
2. weight loss
3. anaemia
4. altered host metabolism

5. muscle weakness

6. malabsorption and diarrhoea

Cachexia is not directly correlated with food intake or type, size or site of the tumour. It may occur with a small primary tumour and precede the clinical diagnosis. Many theories have evolved on the pathogenesis of cancer cachexia:

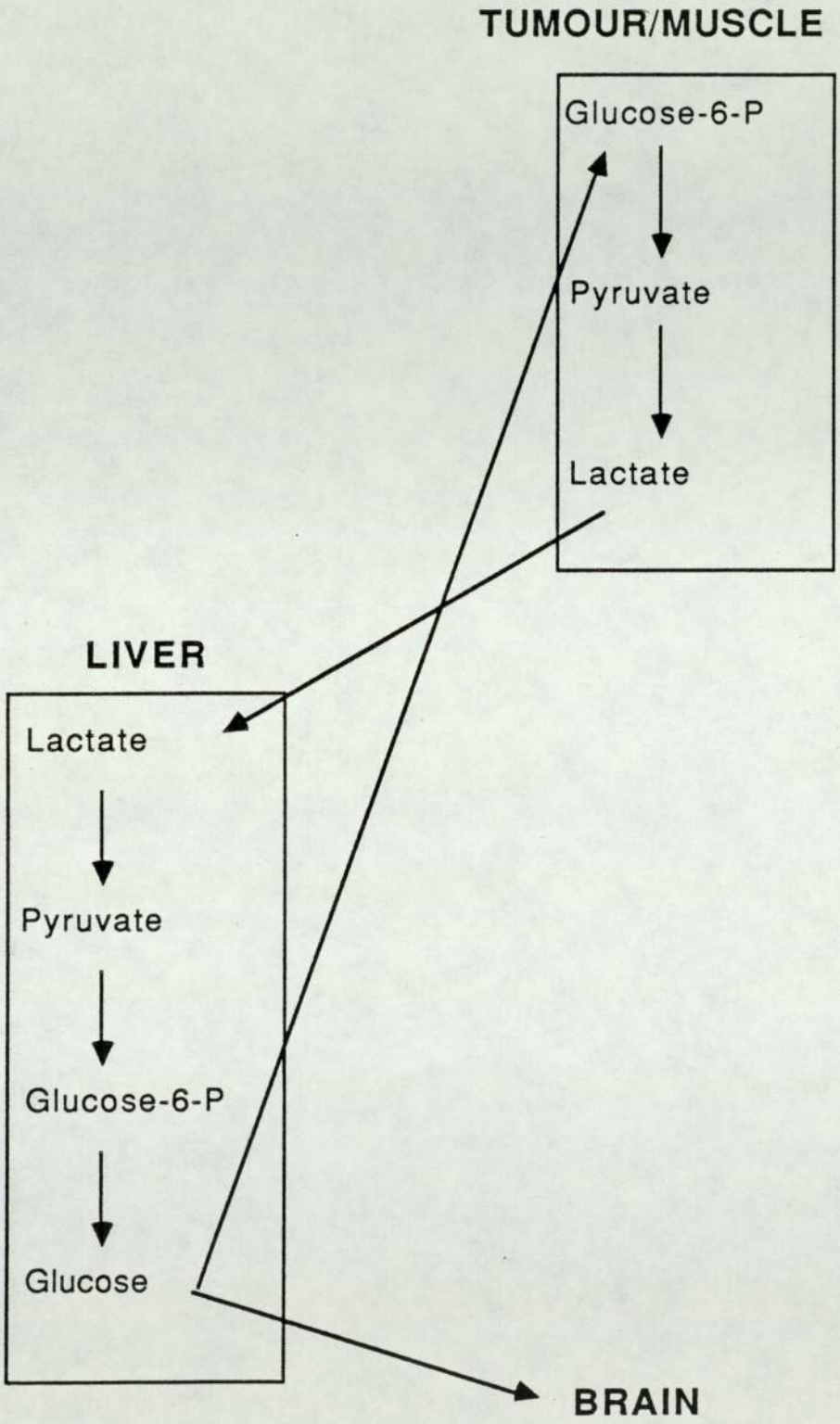
INCREASED ENERGY EXPENDITURE
increased Cori cycle activity
increased nutrient requirement by the tumour
increased gluconeogenesis

ANOREXIA
decreased food intake

ALTERATIONS IN METABOLISM
tumour by-products
synthesis of peptides/biochemical substances

Gold in 1968 concluded that the energy expenditure due to excessive gluconeogenesis by the host to support the tumour produced the cachectic state. The increase in Cori cycle activity (fig 2), results in the tumour gaining two molecules of ATP at the expense of six molecules of ATP to the host used to convert lactate back to glucose. As the tumour grows it consumes increasing amounts of glucose and the energy resources of the host become increasingly depleted by its attempts to maintain a normal blood glucose

Figure 2



THE CORI CYCLE

level. This hypothesis does not explain how small tumours produce cachexia. In addition some tumours can produce cachexia in their hosts but do not have an increased Cori cycle activity (Ainsenberg 1961).

Theologides in 1972 hypothesised that the tumour produces metabolites which activate enzymes in the tissues of the host to cause the cachectic state. He also proposed the production of anorectic peptides which are interpreted by the brain. However to date no substances with these properties have been identified. Following this hypothesis if the satiety signals were blocked then the cachexia should be reversed. However, destruction of the ventromedial hypothalamus, the location of the satiety centre, does not prevent cachexia in tumour bearing animals (Liebelt et al 1971).

Other reports have explained cachexia by a competition for nutrients between the tumour and the host with the tumours needs being preferential to the hosts needs (Stein 1978). Again this does not explain why small tumours cause cachexia in cancer patients. Cachexia can appear in patients with tumours of less than 0.01% of their total body weight (Lindsey 1986).

Evidence that both anorexia and cachexia are the result of circulating tumour by-products is found from parabiotic experiments illustrating that when animals are linked via the circulation cachexia can be conferred on to a non cachectic animal from a cachectic animal (Norton et al 1985). These peptides could be anorexigenic factors, lipid mobilizing factors or other factors that alter host

metabolism. It has been hypothesised that the synthesis of peptides by the tumour results from impairment of repression of selected genes (Cox et al 1978). This explains why some cancer patients never experience cachexia, as the impairment of gene repression does not occur, or they do not possess the genes required to produce the tumour peptide. However, this has still to be proven and it is plausible that no single theory can explain all the observed cases of cachexia.

1.4.2 Cachexia in trauma

There is now a better understanding of cachexia associated with trauma. Weight loss and fatigue are one of the common features found in the trauma due to an injury or a major illness. Weight loss can be as great as 30% after multiple fractures, whereas after uncomplicated surgery only a 6% weight loss occurs (White, Middleton, Baxter 1984). In this cachexia there is an intial shock/stress phase which involves a release of adrenaline. The injury or insult itself then stimulates the hypothalamus and hindbrain to release a number of hormonal releasing factors. These then act on the pituitary to release many hormones such as ACTH, prolactin and growth hormone. Therefore the weight loss produced is associated with a massive increase of normal hormonal effects causing a general mobilization of metabolizable substrates such as the breakdown of triglycerides from adipose tissue.

After this period of depressed metabolism there follows a hypermetabolic stage which can last for many weeks. This

is characterised by an increased heat production and respiration. These effects are further accentuated in patients suffering multiple burns where there is a considerable evaporative heat loss. In burns patients further weight loss is experienced due to vast amounts of water loss. The resting metabolic expenditure can be increased by 50-100% in patients with major burns. This high metabolic rate results in fat and protein breakdown to supply the extra glucose requirement. In a patient with major burns secondary infections may also be involved and their total nitrogen loss may be >300g. This loss of lean body mass can be the cause of death (White, Middleton, Baker 1984).

During these two phases the weight loss experienced is mainly due to an increase in normal hormonal release and a hypermetabolic rate. Specific nutritional support is critical for recovery during these phases and for replacement of lean body mass.

1.5 Metabolism in starvation and cancer cachexia

The major difference seen clinically between starvation and cancer cachexia is in the basal metabolic rate. Starvation leads to a hypometabolic rate whereas many cachectic cancer patients have a hypermetabolic rate. Thus energy expenditure is high in the cachectic cancer patient. In the case of the cancer patient the important questions to be answered are a) is the patient starving primarily because of a lack of food intake or an inability to digest and absorb food, and b) does the cancer patient adapt to

starvation as does the normal man? Before addressing the problem of cancer cachexia the effect of simple starvation on the metabolism of humans will be reviewed.

1.5.1 Starvation

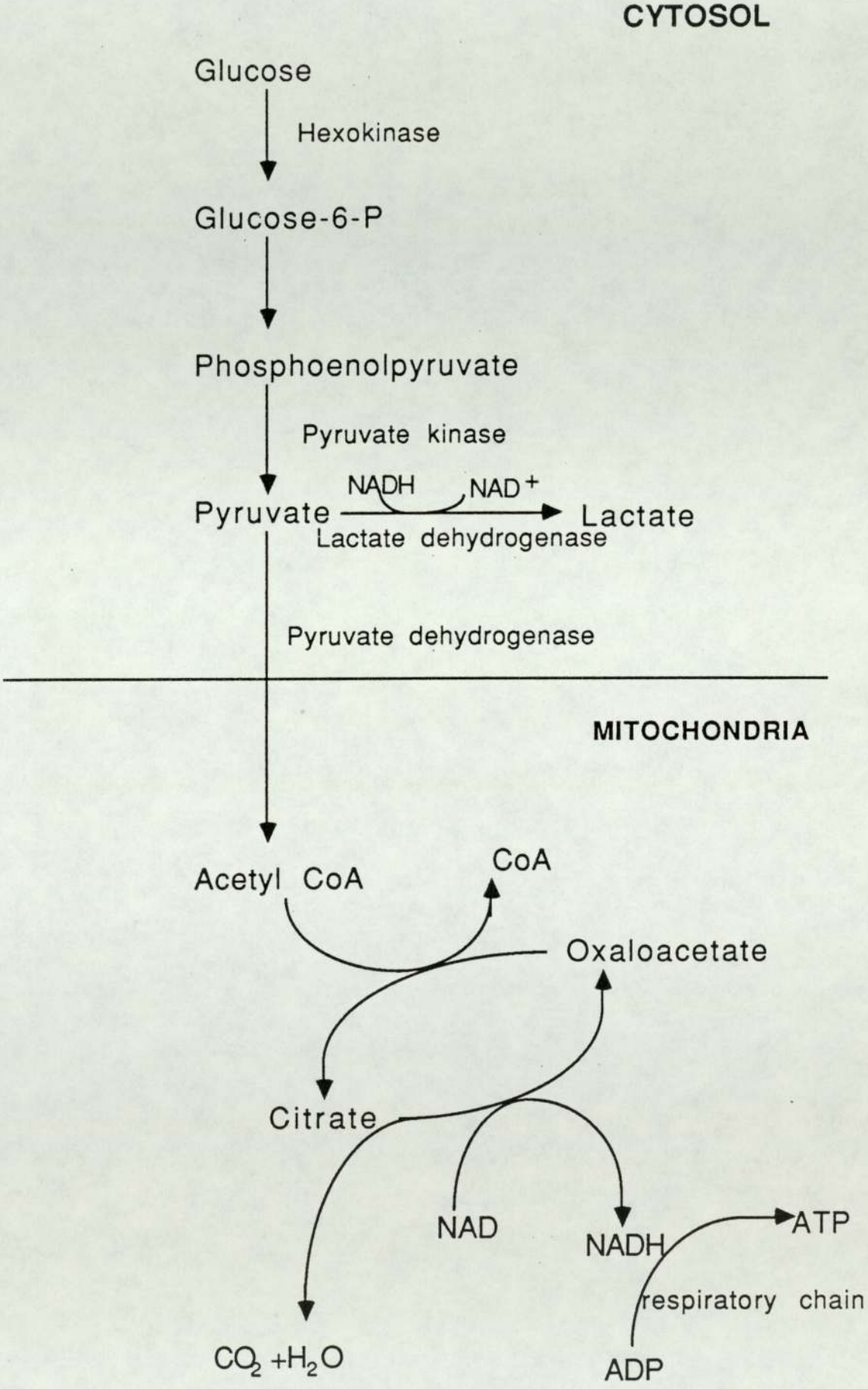
Starvation or fasting is defined as the state of deprivation of food (White, Middleton and Baker 1984). The body fuel reserves of the average 70kg man are as follows:

FUEL	TISSUE	ENERGY kcal
Fat (triglycerides)	Adipose tissue	100000
Carbohydrate		
(glycogen)	Liver	200
	Muscle	400
(glucose)	Body fluids	40
Protein	Muscle	25000
		(<8000 available)

Fat accounts for the majority of fuel reserves and is the main source of energy used in starvation. Protein is less available as the breakdown of more than one third of the bodies protein can lead to death. In a controlled medical study obese volunteers survived fasting for up to six months and more (White, Middleton and Baker, 1984). The first hormonal response to starvation is a fall in plasma insulin and a rise in plasma glucagon concentrations. These events are triggered by a decrease in the blood glucose level. The body then adapts to maintain its blood glucose level to satisfy the glucose requirements of the brain. Glucose is

the major energy source used by the brain. It undergoes glycolysis (conversion of glucose to pyruvate and lactate) and enters the tricarboxylic acid cycle to be ultimately combusted to CO_2 and H_2O (fig 3). The complete oxidation of one mole of glucose by glycolysis and the tricarboxylic acid cycle to CO_2 and H_2O produces 36 moles of ATP. Transient excesses of glucose are converted and stored as glycogen ready to be broken down when required. The supply of glucose is maintained by 3 mechanisms: 1. the conversion of glycogen to glucose in the liver (glycogenolysis), 2. production of glucose from precursors (gluconeogenesis) (fig 4), and 3. a decrease in glucose oxidation by the muscle and other peripheral tissues capable of using alternative fuels. In acute starvation there is a rapid proteolysis with mobilisation of amino acids from muscle, an increase in gluconeogenesis in the liver and the kidney and an increased excretion of urinary urea (Tisdale, 1982). To allow all the available glucose to be used by the brain other peripheral tissues adapt to use other fuels. Alternative fuels used in starvation are excess free fatty acids and ketone bodies (fig 5). The liver responds to the rise in glucagon/insulin ratio by increasing oxidation of fatty acids leading to a significant ketogenesis (fig 6). Ketone bodies produced by the liver readily diffuse into the blood and are carried to extrahepatic tissues where they are converted back to acetyl CoA and then follow the tricarboxylic acid cycle to CO_2 and H_2O. These intial effects last for three to seven days after which the body adapts to conserve protein and so avoid a life threatening catabolism of proteins.

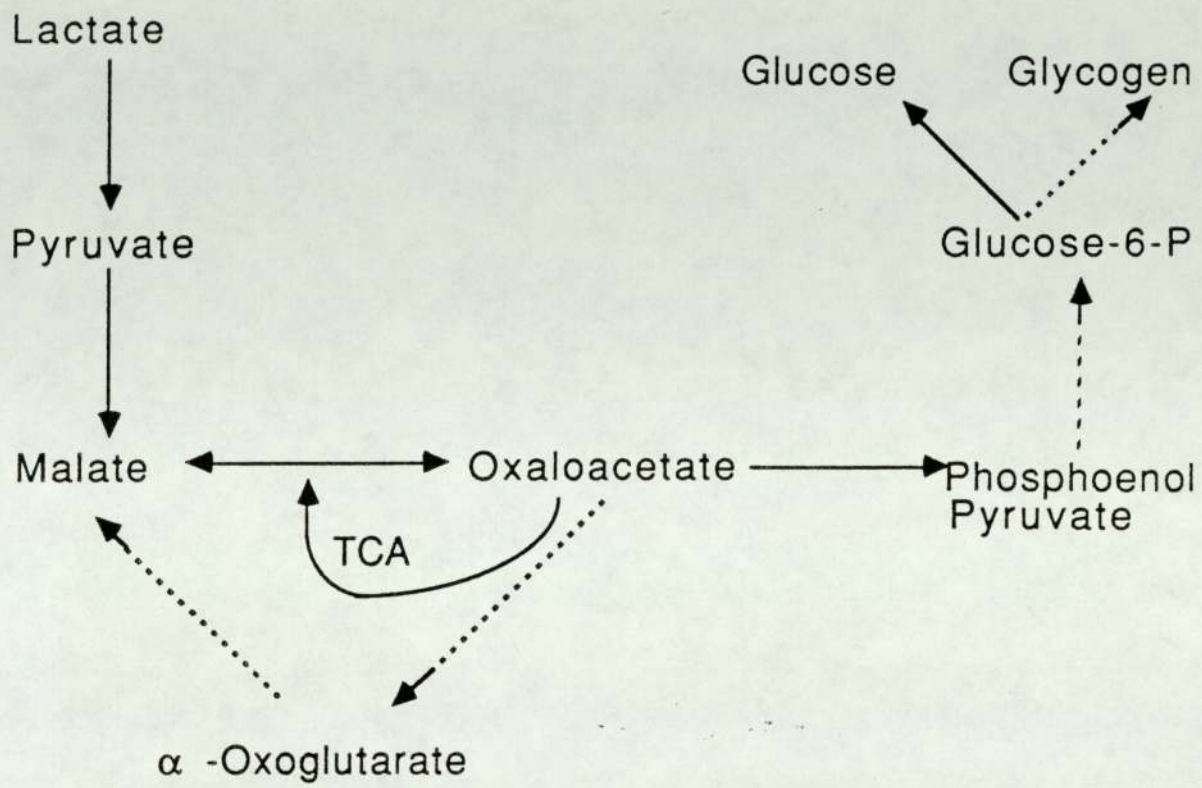
Figure 3



Summary of glycolysis and oxidative phosphorylation

(Tisdale 1986)

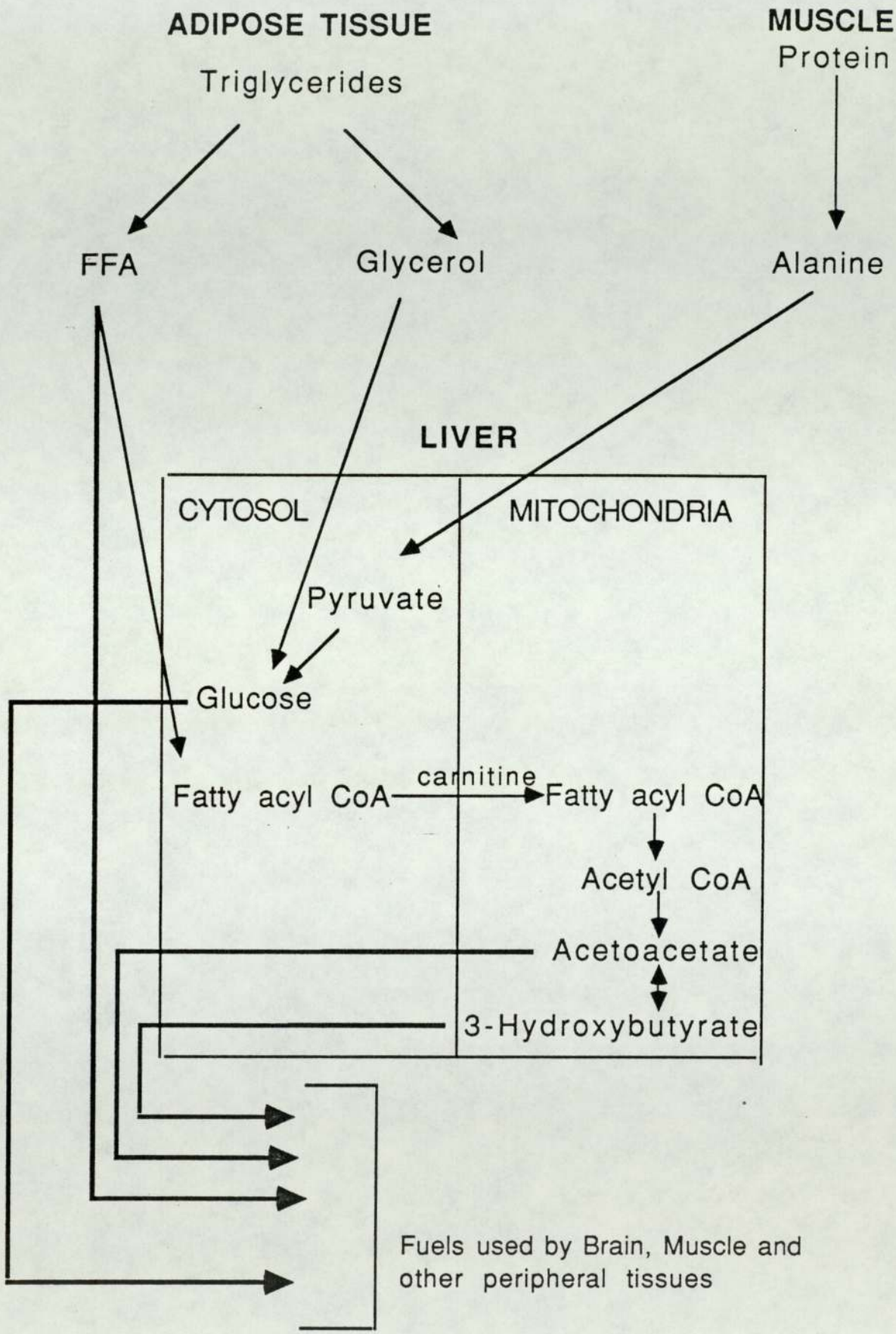
Figure 4



Summary of gluconeogenesis

(Bowman and Rand 1980)

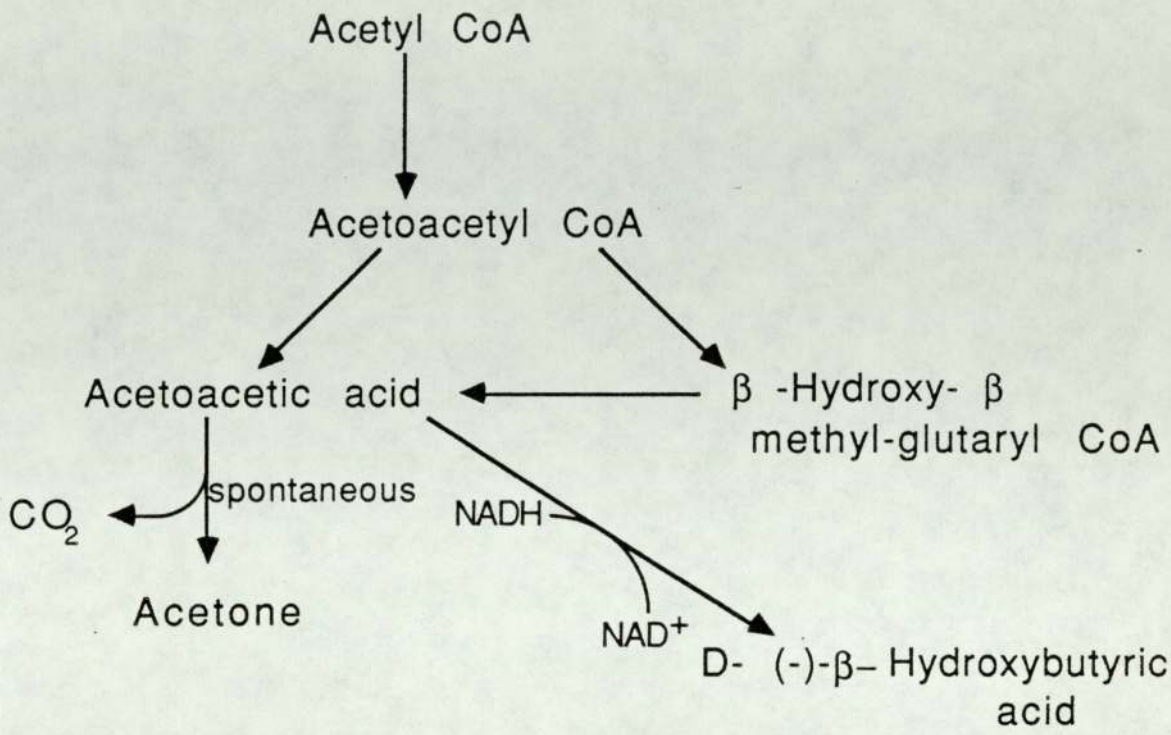
Figure 5



Fuel sources under normal starvation conditions

(Adapted from Tisdale 1986)

Figure 6



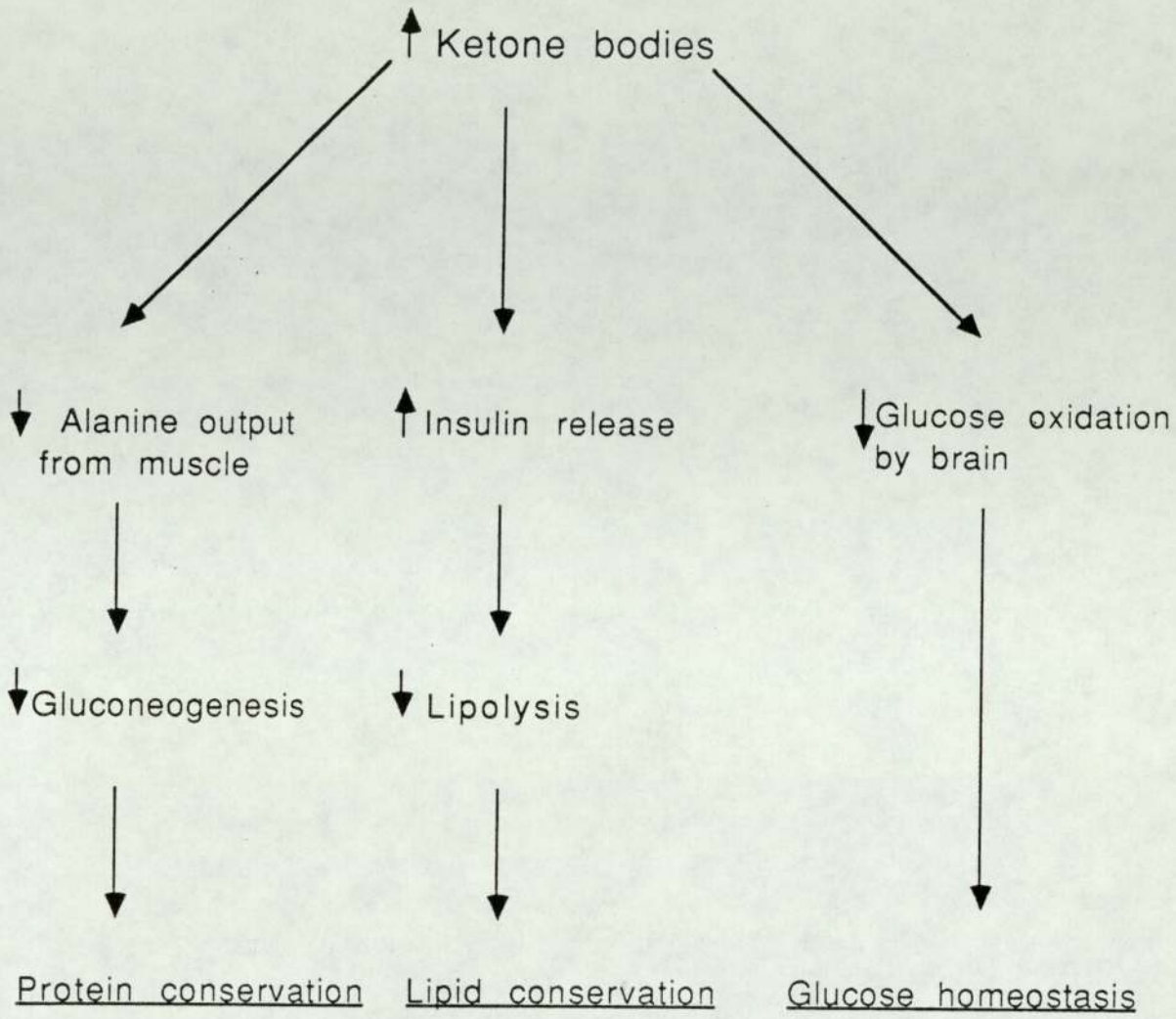
Formation of ketone bodies from acetyl CoA

(Bowman and Rand 1980)

In the latter phase of starvation the brain also adapts so it too can use ketone bodies as an energy fuel (Owen et al 1967). How the brain adapts is unknown but it may involve an increase in activity of ketone body metabolising enzymes and transport system for ketone bodies into the brain. During starvation renal tubular reabsorption of ketone bodies occurs to prevent loss in urine which increases their plasma concentration and thus circulates more to the brain. After a month of starvation ketone bodies will supply 50-60% of the brains energy requirements. This leads to a decrease in overall glucose requirements and a decrease in gluconeogenesis from alanine and lactate in the liver. High ketone body levels also stimulate secretion of the potent antilipolytic hormone insulin from the pancreas (Hawkins et al 1971). Ketone bodies have been shown to directly reduce lipolysis in adipose tissue (Bjorntorp 1966) (fig 7).

By these adaptions a chronically starving man is now living off his major source of stored energy, fat, and will lose about 2% of total body muscle mass in twenty days.Once the body adapts a man can survive for longer periods of time in this fasting state (Brennan 1977).

Figure 7



Role of ketone bodies in prolonged starvation

(Adapted from White, Middleton and Baker 1984)

1.5.2 Cancer cachexia

For a better understanding of the weight loss experienced by cancer patients it is also necessary to understand both tumour and host metabolism. The tumour and host must be considered metabolically as two separate identities (Costa 1963).

1.5.2.1 Tumour metabolism

Tumours display a high aerobic glycolysis which may relate to the malignant state. Radiotherapy studies have shown that large solid tumours have a poor blood supply and contain many hypoxic tumour cells. Hence glucose is the only substrate the tumour can utilize in these conditions via the Embden-Meyerhoff pathway to supply the energy it needs for growth in the absence of oxygen (Tisdale, 1986). The increased glycolytic activity leads to large amounts of lactic acid produced by the tumour. In the Walker 256 rat carcinoma the pH decreases from 7.3 to 6.2 with increasing tumour mass up to 50g (Jain et al 1984). Ehrlich ascites tumour cells have shown a high activity of the enzyme lactate dehydrogenase (Lazo and Sols 1980). This may be the reason for their increased glycolytic activity. The nutritional requirements of the tumour reflects on host metabolism and may cause the changes in body composition seen in cachexia.

1.5.2.2 Host metabolism

Cachectic cancer patients experience changes in carbohydrate, lipid and protein metabolism.

A. Carbohydrate metabolism

The tumour has a vast requirement for glucose which leads to a strain on the hosts ability to maintain normal blood glucose levels. Thus gluconeogenesis from glycerol is increased in cancer patients (Lundholm et al 1982). The drug clofibrate, which lowers plasma levels of triglycerides, has been shown to reduce the growth of Walker 256 carcinosarcoma in rats (Gold 1978). Thus lowering the levels of glycerol available for gluconeogenesis reduces the glucose available to feed the tumour. An increased Cori cycle activity (conversion of lactate to glucose in the liver) has been found in patients with metastatic carcinoma and progressive weight loss (Holroyde et al 1975). Gluconeogenesis from alanine is also increased in cancer patients (Waterhouse et al 1979). Thus there is an overall increase in gluconeogenesis to supply the glucose required to maintain normal blood glucose levels in the host. Glucose intolerance is seen in cancer cachexia due to an abnormal insulin production and insulin resistance (Kisner 1978). The use of insulin in the nutritional management of cachectic cancer patients has been suggested (Schein et al 1979) and is discussed further in section 4.7.

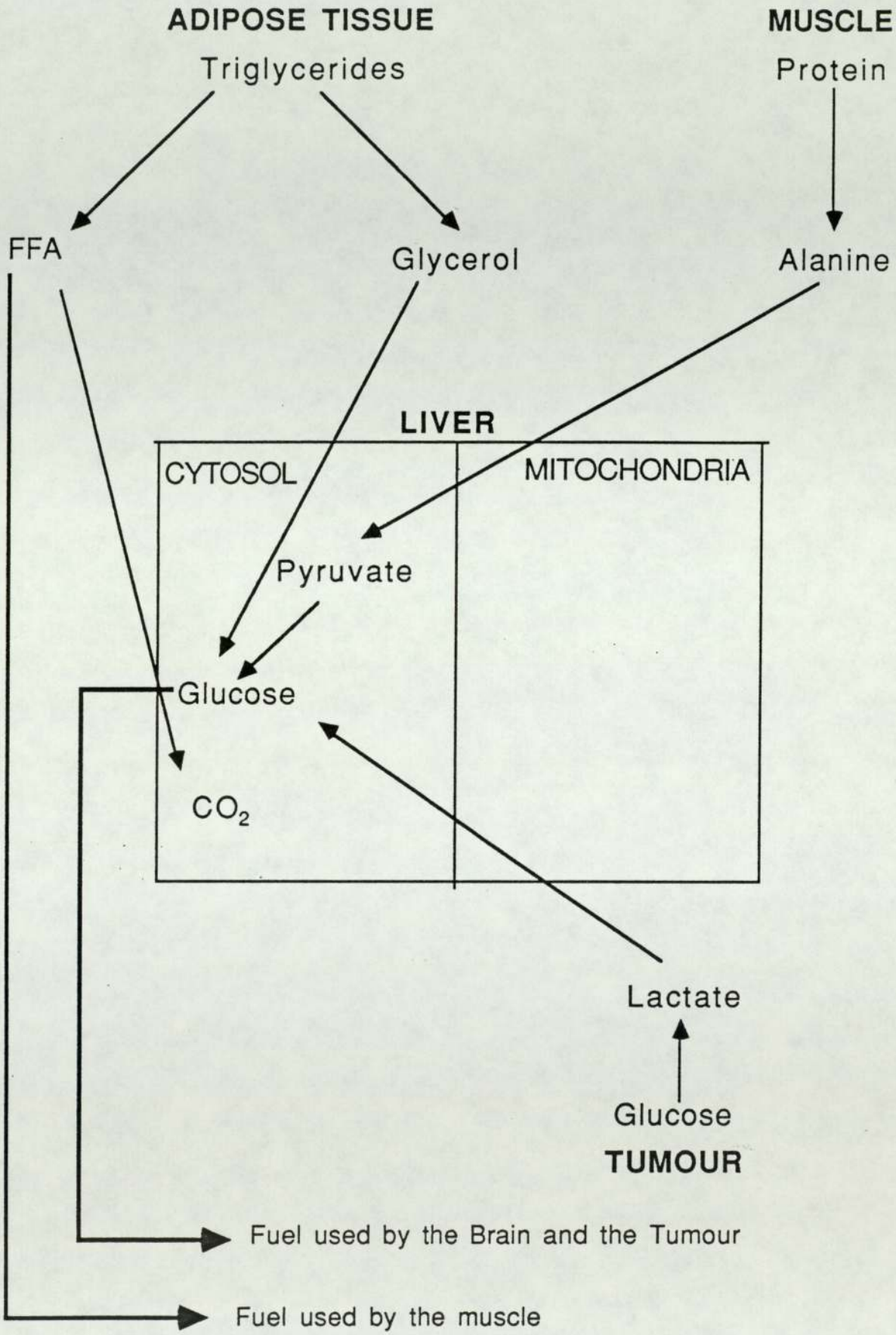
B. Lipid metabolism

A progressive decrease in carcass lipids is an important feature of cachexia in both animals and humans. This is due to mobilization of free fatty acids from the hosts adipose tissue, and occurs even when the tumour mass is small. Frederick and Begg in 1956 demonstrated that the

weight loss seen in tumour bearing animals was a result of a decrease in body fat directly associated with an increase in plasma free fatty acids. Recently lipolytic substances produced by the tumour have been identified, which appear to act directly on adipose tissue. One example is Toxohormone-L which has been isolated from the ascites fluid of mice with sarcoma 180 and patients with hepatoma, Grawitz tumour, ovarian tumours and from the pleural fluid of patients with malignant lymphoma (Masuno et al 1981). Another is cachectin which has been isolated from endotoxin stimulated macrophages (Beutler et al 1985). This peptide inhibits lipoprotein lipase and it has been suggested that cachexia is mediated via this action. The effect of cachectin in cachexia is further reviewed in section 4.2.

As stated previously ketosis is a common phenomena in starvation; however ketosis in cancer cachexia is very rare. Ketonuria is uncommon in cancer patients (Conyers et al 1979) and in tumour bearing rats (Mider 1951) although extensive weight loss still occurs due to mobilisation of FFA from adipose tissue (fig 8). If these patients are given an exogenous supply of FFA ketonaemia is observed. Thus there is no deficiency or impairment of the livers ability to synthesize ketone bodies (Magee et al 1979, Conyers et al 1979). This absence of ketosis can explain the loss of muscle protein, breakdown of adipose tissue and decreased insulin secretory capacity seen in cachectic cancer patients. Many tumours have shown to be deficient in enzymes that utilise ketone bodies as an energy fuel, for example a range of animal and human tumours of peripheral

Figure 8



Fuel sources in cachectic cancer patients

(Adapted from Tisdale 1986)

tissues have been shown to have low or no activity of 3-oxoacidCoAtransferase (Tisdale and Brennan 1983). Thus it may be possible to selectively starve the tumour with a ketogenic regimen whilst at the same time promoting maintenance of host proteins.

C. Protein metabolism

Another characteristic of cancer cachexia is a loss of skeletal muscle mass. This can be one of the most devastating effects produced in cachexia. In tumour bearing rats, skeletal muscle protein synthesis is reduced and protein synthesis is depressed by 70% in muscle and by 40% in the liver in cachectic tumour bearing mice (Emery et al 1984). At the moment it is not known to what extent a decreased rate of synthesis or an increased rate of catabolism contribute to the depletion of muscle protein. Tumour cells have the ability to concentrate amino acids from the plasma pool in preference to normal cells. The tumour acts as an nitrogen trap competing with the host for nitrogen compounds. Thus a negative nitrogen balance has been shown in a variety of animal and human tumours (Theologides 1972). An amino acid imbalance can arise in the host due to the tumour preferentially taking one or more of the essential amino acids it requires from the hosts pool. Decreased plasma levels of serine, glycine, aspartate and hydroxyproline were found in rats bearing the Walker 256 carcinosarcoma (Krause et al 1979). In theory the addition of the depleted amino acids should correct the imbalance caused by the tumour and limit the cachexia. Some tumours

cannot synthesize some non essential amino acids, for example some leukaemic cells cannot synthesize L-asparagine. Administration of the enzyme L-asparginase will deplete the host of asparagine and so decrease the effect the tumour has on the host. Therefore, if the tumour is dependent on a certain amino acid again it is possible to selectively starve the tumour.

Hence cancer cachexia may arise from the biochemical requirements imposed on the host by the tumour or circulatory catabolic factors produced by the tumour or associated macrophages. A better understanding of the host-tumour interaction will help in the development of treatments for cancer cachexia.

1.6 Nutritional therapy of cachexia

Nutritional support of the cancer patient is used for three purposes 1) to reverse the cachectic state, 2) to prevent weight loss and 3) to improve the host tolerance to chemotherapy, radiotherapy or surgery (Calman and Fearon, 1986). Response to nutritional supplementation in the cachectic cancer patient appears less than the response achieved in the malnourished patient (Nixon et al 1981a). Thus increasing food intake does not completely abolish the cachectic syndrome. When weight gain is acheived it is usually due to fat deposition rather than a repletion of vital lean body mass (Cohn et al 1982). Nutritional support can be given enterally or parenterally and can range from simple dietary advice to hospitalization and total parenteral nutrition. The benefits of this type of

treatment have to be considered against any detrimental effects to the patient. Clinical indices that prompt nutritional support are (i) a weight loss in excess of 10% of preillness weight, (ii) a decrease in serum albumin to less than 3.5g%, (iii) a decrease in the creatinine-height ratio to less than 80% of the normal value and (iv) a demonstrated immune competence (Harvey et al, 1979 and Hickman et al, 1980). The type and amount of nutritional support will be determined by the degree of weight loss, progression of disease, ultimate prognosis and the type of treatment the patient is receiving.

1.6.1 Diet

The easiest way to provide nutritional support is by the patient eating voluntarily. Unfortunately anorexia is a common phenomemna seen in cancer patients (Dewys 1979). In many cancer patients taste abnormalities have been reported and this may be due to an elevated threshold for sweetness (Dewys 1977). Thus these patients may benefit from increased seasoning or flavouring of their food. If vomiting is a problem standard antiemetics may be prescribed. Another method to increase oral intake is education of the patient to establish a target weight , to measure and provide regular feedback. Regaining lost weight is often not possible and so the maintenance of the patients current weight is the chosen target. Milk-based oral food supplements are available that are high in calorific value. These supplements have a high protein content and will help the patient regain weight. Many commercial products are now

in retail stores, for example Complan. In patients where an increase in oral intake is possible, a dietician assesses their nutritional status and instructs them on how to eat a balanced diet.

1.6.2 Enteral hyperalimentation

If the cancer patient is unable to consume enough food on a voluntary basis nasogastric feeding may be used to supply nutritional support. The nasogastric tube by passes the mouth, oesophagus and stomach and supplies nutrients straight into the gastrointestinal tract. Diets used in these tubes are usually one of four types (Calman and Fearon, 1986)

1. Blenderized diets: these are prepared by liquidizing ordinary foods and contain whole protein , carbohydrates and a mixture of fats.
2. Milk-based diets: these diets are high in protein and other nutrients found in milk.
3. Lactose-free/whole protein diets: these are commercially produced diets containing egg albumin, soy or caesin as the protein source.
4. Elemental diets: these diets are formulated synthetically out of chemical nutrients for example Vivonex. These diets do not need proteolytic or lipolytic enzymes for digestion and so are useful in patients who have impaired gastrointestinal function.

The side effects of nasogastric feeding are generally mild. Some breathing difficulties may be encountered when the patient is wearing the nasogastric tube. Other more

common side effects are diarrhoea and metabolic irregularities due to dehydration and electrolyte imbalance. However, in many patients nasogastric feeding is well tolerated.

1.6.3 Parenteral hyperalimentation

When the gastrointestinal tract cannot be used to provide adequate dietary intake, nutrients may be administered intravenously. Peripheral veins are not used for this purpose due to a high incidence of thrombophlebitis. Total parenteral nutrition (TPN) is administered via the central venous system. A major disadvantage of this technique is that the financial cost is high. Thus TPN is not used without careful consideration of the advantages and disadvantages to that particular patient. Of 119 small-cell lung cancer patients, 66 had a prediagnosis weight loss greater than or equal to 2% of their original body weight and were considered to be malnourished. Of these 32 received TPN for four weeks whilst the others continued with normal oral intake (Hardy et al 1986). This treatment, however, did not improve survival or response to chemotherapy. With further knowledge of metabolic alterations in cancer cachexia concern has been raised that hyperalimentation may accelerate tumour growth by selectively feeding the tumour. Hyperalimentation has been shown to stimulate the growth of several experimental tumours (Balducci and Hardy 1985). Patients with metastatic colorectal cancer were randomised to receive hyperalimentation or to eat a regular diet. In

the patients with liver metastases hyperalimentation decreased survival. However no increase in tumour growth was noted (Nixon et al 1981b). Holroyde and Reichard (1981) found increased serum levels of lactic acid during nutritional support. The tumour is the main producer of circulatory lactic acid in the cancer patient and therefore this suggests a stimulation of tumour growth. Hence it is important that nutritional support be less aggressive since the increased nutrition may accelerate tumour metabolism to the detriment of the patient (Dewys 1985). All these factors must be considered before any nutritional support is administered to the cachectic cancer patient.

1.6.4 Future therapy

A better understanding of the cachectic mechanisms occuring in the cancer patient may improve the future therapy of this syndrome. Contrary to hyperalimentation a new approach is to starve the tumour whilst preferentially feeding the host. The observation that tumours do not metabolise lipids (Weber 1982) to any extent has lead to the idea that a diet high in lipids may starve the tumour whilst being able to feed the host. Ketone bodies maintain homeostasis in starvation and so may be able to reverse some of the cachectic effects seen in cancer patients. Most solid tumours are unable to utilise ketone bodies as an energy source due to enzyme deficiencies (Tisdale and Brennan 1983). This again provides a way of starving the tumour whilst feeding the host (Tisdale 1986).

Recent discoveries of circulatory catabolic factors

produced by the tumour may lead to a new approach to the treatment of cancer cachexia. In the future these factors could be isolated, inhibitors synthesized, and these inhibitors administered to the cancer patient as a treatment for cachexia.

1.7 Aims of this investigation

Cachexia, a serious and potentially lethal consequence of cancer, has been reviewed in section 1.0. Cancer cachexia has previously been investigated using various experimental tumour models. These animal models were not analogous to the human situation as cachexia in many of the models used did not appear until the tumour burden was large. The initial aim of the work described in section 4 was to establish whether the MAC16 adenocarcinoma when transplanted in NMRI mice, is a suitable model of the cachexia witnessed in cancer patients. This was investigated by measuring the weight loss and body composition in tumour bearing mice and determining their food and water intake. The metabolic effect of the tumour on the host was investigated by monitoring the levels of various plasma metabolites and of nitrogen excretion.

Once the MAC16 adenocarcinoma was established as a good model of cachexia, the mechanism by which cachexia is caused in this model was investigated. Evidence has been reported indicating that some mammalian tumours produce catabolic factors which may be responsible for the cachectic condition of the host (Kitada et al 1980, Masuno et al 1981). The primary aim of this study was to detect the presence of any

catabolic factors produced by the MAC16 tumour. Two in vitro assays were used to measure the lipolytic and proteolytic activity in the MAC16 tumour and that released into the circulation of the host.

Catabolic factors detected were characterised by both the effect of numerous catabolic enzymes and inhibitors on their activity and by gel filtration. Using these methods the possible presence of similar catabolic factors was investigated in non cachexia-inducing tumours and in the circulatory body fluids of both patients with cancer or with other weight losing diseases, to determine whether these factors were present only in cachectic tumours.

The mechanism of action of catabolic factors released by the MAC16 adenocarcinoma was further studied using three separate approaches. (i) The role of endogenous second messengers in the action of these factors. (ii) The effect of various drugs and dietary treatments were considered in their abilities to reduce or even abolish the cachexia induced by the MAC16 adenocarcinoma. (iii) Relationship between inhibition of tumour cachectic factors and tumour growth.

The overall aim of this investigation was to increase the knowledge of the etiology of cachexia in cancer, to provide a better understanding of the effects the tumour has on its host and to investigate possible treatments for use in eleviating cancer cachexia as well as new approaches in the treatment of solid tumours.

SECTION 2: MATERIALS

2.1 ANIMALS

Pure strain male/female NMRI, Balb/C, BKW and MF1 mice (age 12 - 15 weeks) were purchased from Bantin and Kingman, Hull, UK. Rat and mouse breeding diet was purchased from Pilsburys Ltd, Birmingham, UK.

2.2 CHEMICALS

The following compounds were obtained from:

ALPHA LABORATORIES LTD, Hampshire, UK.

Wako NEFA C kit

AMERSHAM INTERNATIONAL, Amersham, Bucks, UK.

cAMP kit

ASDA SUPERSTORE, Birmingham, UK.

Corn oil (100% pure)

BDH LIMITED, Poole, England.

Ethylenediaminetetraacetic acid, disodium salt

Potassium hydroxide

Sodium hydroxide

Trichloroacetic acid

BIO-RAD LABORATORIES, Richmond, CA.

Biogel P2

Biogel P4

Bradford reagent

BOC LTD, London, UK.

Nitrous oxide

Oxygen

EVANS MEDICAL LTD, Greenford, Middlesex, UK.

Isophane insulin

FISONS SCIENTIFIC APPARATUS, Loughborough, UK.

Magnesium sulphate

2-methoxyethanol

FSA LABORATORY SUPPLIES, Loughborough, UK.

Acetone

Acetonitrile

Chloroform

Diethylether

Ethanol

Hydrochloric acid

Perchloric acid

Propanol

Sodium chloride

GIBCO LTD, Paisley, Scotland.

RPMI 1640 medium

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

Halothane

IMPERIAL LABORATORIES LTD, Andover, Hants, UK.

Foetal calf serum

PILSBURYS LTD, Birmingham, UK.

Dicalcium carbonate

Rat and mouse breeding diet

Rodent 006 premix

Sodium caesinate

Soya

SIGMA CHEMICAL CO, Dorset, UK.

Acetoacetic acid, lithium salt

Acid phosphatase

Adenosine-5-triphosphate, trisodium salt

Adrenocorticotrophic hormone

Alanine

Alkaline phosphatase

Antitrypsin

Bentonite

Calcium carbonate

Collagenase

Creatinine kit

Docosahexaenoic acid

Eicosapentaenoic acid

Glycerokinase

3-Hydroxybutyrate dehydrogenase

Indomethacin

Isobutylmethylxanthine

Lactate dehydrogenaseNicotinamide adenine dinucleotide

L-Methionine

Nicotinamide adenine dinucleotide reduced form

Ninhydrin reagent

O-Toluidine kit

Palmitic acid

Phenylmethylsulphonylflouride

Phosphoenolpyruvate

Prostaglandin E_1

Prostaglandin E_2

Prostaglandin F_1

Prostaglandin F_2

Salbutamol

Sephadex G50, G150, G25

Sodium butyrate

Sodium 3-hydroxybutyrate

Sodium periodate

Sulphatase

Triethanolamine

Triflouroaceticacid

Triglyceride kit

Urea/ ammonia nitrogen kit

SCIENTIFIC HOSPITAL SUPPLIES LTD, Liverpool, UK.

Liquigen Medium chain triglyceride solution

WEDDEL PHARMACEUTICALS LTD, Wrexham, UK.

Multiparin (Heparin)

WHATMAN BIOSYSTEMS LTD, Maidstone, Kent, UK.

DE52

2.3 GIFTS

The following compounds were kindly donated by:

M.C.M. KLOSTERFRAU, Koln, Germany.

Fish oil

DR J. DOUBLE, University of Bradford.

Flavone acetic acid

SOLVAY ET CIE, Brussels, Belgium.

3-hydroxybutyrate, arginine salt

BRISTOL MYERS, Evansville, USA.

Megace

BOEHRINGER INGLEHEIM, Bracknell, Berks, UK.

Tumour necrosis factor

2.4 BUFFERS

16 mM Tris- 0.32 mM hydrazine hydrate buffer, (pH 8.5)

Hydrazine hydrate	2 ml
0.1 M Tris- HCl buffer (pH 8.5)	40 ml

The pH was adjusted to pH 8.5 with concentrated hydrochloric acid and the final volume was made up to 50 ml with 0.1 M Tris-HCl buffer (pH8.5).

0.1 M Tris- HCl buffer (pH 8.5)

Trizma base	6.05 g
Distilled water	400 ml

The pH was adjusted to pH 8.5 with concentrated hydrochloric acid and the final volume was made up to 500 ml with distilled water.

0.1 M Phosphate buffer (pH 6.8)

Solution A: Potassium dihydrogen orthophosphate (13.609 g) was added to 1000 ml of distilled water.

Solution B: Dipotassium hydrogen orthophosphate, trihydrate (22.82 g)was added to 1000 ml of distilled water.

Solution B was added to 200 ml of solution A until a pH of 6.8 was reached.

0.01 M Phosphate buffer (pH 8.0)

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

Solution B: Dipotassium hydrogen orthophosphate, trihydrate (2.28 g) was added to 1000 ml of distilled water.

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

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

Hydrazine hydrate	25 ml
Glycine	11.4 g
Distiiled water	200 ml

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

Krebs-Ringer bicarbonate buffer (pH 7.6)

0.9% Sodium chloride	100 ml
1.15% Potassium chloride	4.0 ml
1.22% Calcium chloride	3.0 ml
2.11% Potassium dihydrogen phosphate	1.0 ml
3.82% Magnesium sulphate, hydrated	1.0 ml
1.3% Sodium bicarbonate	21.0 ml

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

0.1 M Sodium acetate buffer (pH 4.5)

Sodium acetate	1.3608 g
Distilled water	50 ml

The pH was adjusted to pH 4.5 with glacial acetic acid and the final volume was made up to 100 ml with distilled water.

0.05 M Tris- HCl buffer (pH 7.5)

Trizma base	3.025 g
Distiiled water	400 ml

The pH was adjusted to pH 7.5 with concentrated hydrochloric acid and the final volume was made up to 500 ml with distilled water.

0.2 M Citrate buffer (pH 5.0)

Citric acid	21.008 g
NaOH (1N)	200 ml

The pH was adjusted to pH 5.0 and made up to 500 ml with distilled water.

Ninhydrin reagent

0.8 g of ninhydrin powder was dissolved in 500 ml of citrate buffer pH 5.0. This was then added to 500 ml of 2-methoxyethanol in which 20 g of ninhydrin powder had been dissolved. This solution was protected from light.

SECTION 3: METHODS

3.1 In vivo characterisation of MAC16 colon adenocarcinoma in mice

3.1.1 Transplantation of tumours

The MAC13, 15A and 16 colon adenocarcinomas were originally induced with 1,2-dimethylhydrazine by Dr J. Double, Bradford University (Bibby et al, 1987a). Of these tumours only the MAC16 colon adenocarcinoma showed symptoms of cachexia in the host. The MAC16 adenocarcinoma was excised from donor animals, placed in sterile isotonic saline and cut into small fragments 1 x 2 mm in size. Using a trocar, fragments were implanted subcutaneously into the flank of the right hind limb of NMRI mice (Mr. M. Wynter, Aston University). The doubling time of this tumour was 10 days determined by Mr Mike Wynter, Aston University. Mice were weighed and food and water consumption were measured daily. Before any of the animals had lost more than 30% of their original body weight, they were sacrificed. Carcass and tumour weights were recorded and blood samples collected (see section 3.1.2). The carcasses were stored at -20°C prior to analysis of body composition.

3.1.2 Collection of blood samples

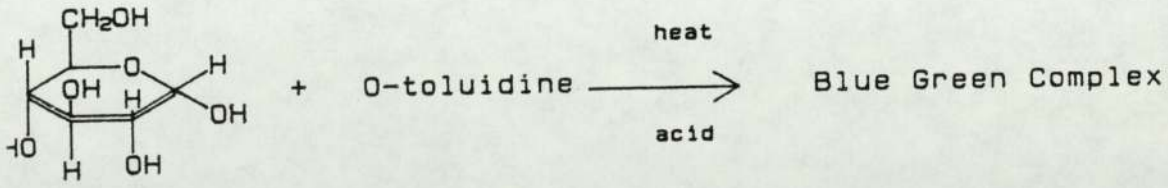
Between 9.30 and 10.30 am blood was removed from mice by cardiac puncture under anaesthesia using a mixture of halothane, oxygen and nitrous oxide. Approximately 1.0 ml of blood was collected from each mouse using a heparinised

syringe. Blood samples were transferred to microfuge tubes on ice and assayed to determine the levels of glucose, free fatty acids, 3-hydroxybutyrate (3-HB), acetoacetate and triglycerides in the plasma (see sections 3.1.3 - 3.1.6).

3.1.3 Determination of blood glucose levels

Glucose levels were determined on whole blood with the use of the Sigma o-toluidine kit (no 635). This kit utilises the methods of Hyvarinen and Nikkila (1962), and Feteris (1965). In the presence of heat and acid, O-toluidine reacts readily with glucose to form a blue-green complex (figure 9). The intensity of the colour, which was proportional to the glucose concentration, was measured at 635 nm using a Beckman DU70 spectrophotometer.

Figure 9



3.1.4 Determination of plasma free fatty acid levels

Plasma was obtained by centrifuging whole blood for 1.0 min in a Beckman microfuge. Free fatty acid levels were measured in 20µl of plasma using a Wako NEFA C kit (Alpha laboratories). The kit utilises an *in vitro* enzymatic colourimetric method following the scheme illustrated in figure 10 (Duncombe, 1964

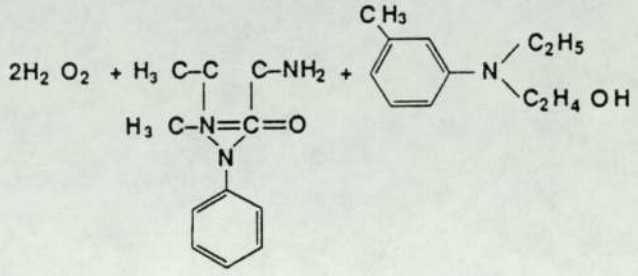
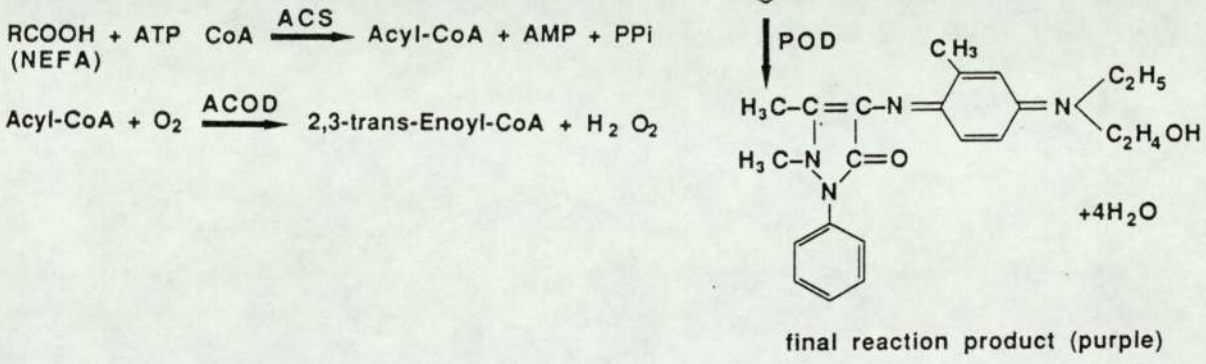


Figure 10

/



3.1.5 Determination of plasma 3-hydroxybutyrate and acetoacetate levels

3.1.5.1 Preparation of blood samples

Whole blood (100µl) was transferred to a microfuge tube on ice and deproteinised by adding 100µl of ice cold 10% w/v perchloric acid. This was mixed thoroughly and centrifuged in a Beckman microfuge for 1.0 min. The supernatant was used as follows for analysis of 3-hydroxybutyrate and acetoacetate.

3.1.5.2 Analysis of 3-hydroxybutyrate levels

The levels of 3-hydroxybutyrate in the plasma were determined using the method of Williamson and Mellanby (1974). This utilises the oxidation of 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase with the concominant reduction of NAD.

$$CH_3CHOHCH_2COOH + NAD^+ \longrightarrow CH_3COCH_2COOH + NADH + H^+$$

The formation of NADH was measured spectrophotometrically at 340 nm. Assay cuvettes contained 16 mM Tris-0.32 mM hydrazine hydrate buffer (pH 8.4), 0.45 mM NAD and 50 µl of sample in a total volume of 3.1 ml. The reaction was initiated by the addition of 10 µl of 3-hydroxybutyrate dehydrogenase (150 mU/ml) and the increase in absorbance was measured for 40 - 60 min at a temperature of 25°C.

3.1.5.3 Analysis of acetoacetate levels

The levels of acetoacetate in the plasma were determined using the reverse of the reaction shown in section 3.1.5.2 (Mellanby and Williamson, 1974). Assay cuvettes contained 33 mM phosphate buffer (pH 6.8), 0.2 mM NADH and 50 µl of sample in a total volume of 3.1ml. The reaction was initiated by the addition of 10µl of 3-hydroxybutyrate dehydrogenase (50 mU/ml). The decrease in absorbance was measured at 340 nm for 20 min at a temperature of 25°C.

3.1.6 Determination of plasma triglyceride levels

The level of triglycerides in the plasma were determined using a Sigma diagnostic kit. The kit utilises the methods of Kessler and Lederer (1966), and Fletcher (1968) and is a colourimetric determination following the scheme shown below:

triglycerides + KOH ------> glycerol + fatty acids

glycerol + periodate ------> HCHO

HCHO + NH_4^+ + acetylacetone ------> diacetyldihydrolutidine

Samples of plasma (50 - 100 µl) were extracted with isopropanol and triglyceride purifier. Aliquots (0.5 ml) were added to 0.125 ml of potassium hydroxide (1N) and incubated at 60°C for 5 min. To these solutions were added 0.125 ml of periodate solution (125mg Sodium periodate in 50ml of 2N acetic acid) followed after 10 min by 0.75 ml of colour reagent consisting of 20 ml of ammonium acetate solution and 40 ml of isopropanol. The samples were covered and placed in a water bath at 60°C for 30 min. The yellow product, diacetyldihydrolutidine, exhibited a maximum absorbance at 410 nm. The absorbance of the samples at this wavelength was proportional to the concentration of triglyceride.

3.1.7 Body composition analysis

The gastrocnemius and thigh muscle were carefully dissected out from the left leg of the carcass and weighed.

Carcass and muscles were placed in an oven at 80°C until a constant weight was reached. Dry weights of the carcass and muscles separately were recorded. The water content for the muscle and total carcass was then calculated from the wet and dry weights. Total fat content of the carcass was determined using the method of Lundholm et al (1980). Each carcass was broken up into small pieces and then extracted in turn with 25 ml of acetone:ethanol (1:1 v/v), chloroform:methanol (1:1 v/v) and diethylether. The extracts were combined in a preweighed round-bottomed flask. The solvents were then removed under vacuum using a Buchi rotary evaporator to leave a fatty residue. The flask was reweighed and the total fat content per carcass calculated.

3.1.8 Urine analysis

3.1.8.1 Metabolic cage experiments

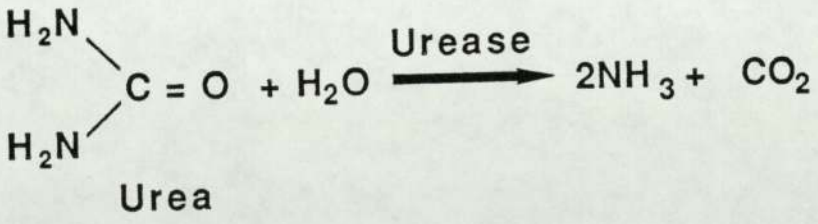
Urine was collected from mice for 24 h in a metabolic cage (Jencons). Water and food was available ad libitum and the amount of food eaten was measured so nitrogen intake could be calculated. The apparatus was set up with a mechanical pump to circulate air throughout the cages. At the end of the 24 h period the urine was stored at -20°C prior to determining the levels of urea, ammonia and creatinine (see sections 3.1.8.2 and 3.1.8.3). An aliquot of urine (100µl) was also used to determine the presence of lipolytic factors as described in section 3.4. In the weight loss and nitrogen output studies the same mice were

put back into the metabowls at varying intervals (usually consecutive days). This allowed nitrogen excretion to be determined at varying amounts of weight loss. Weight loss was compared to nitrogen excretion in the same animals. Control animals with no tumours were also put into the metabowls on the same days but showed no difference in nitrogen excretion with time.

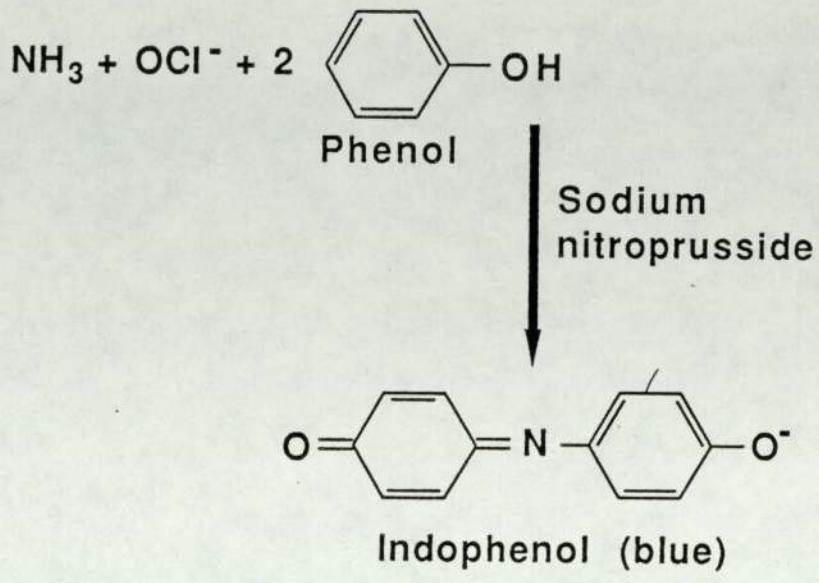
3.1.8.2 Urea/ammonia determination

Urea/ammonia nitrogen were determined/ using a Sigma diagnostic kit which utilised the methods of Fawcett and Scott (1960) and Chaney and Marback (1962). This procedure was dependent upon the action of urease to produce ammonia:

Figure 11



The ammonia was reacted with alkaline hypochlorite and phenol in the presence of a catalyst, sodium nitroprusside, to form indophenol:



The concentration of ammonia was directly proportional to the absorbance of indophenol which was measured spectrophotometrically at 570 nm.

3.1.8.3 Creatinine determination

Creatinine was determined using a Sigma diagnostic kit. This method of creatinine measurement was an improved method based on the Jaffe reaction, where yellow/orange colour forms when the metabolite is treated with alkaline picrate . The method was improved by Slot (1965) who noted that under acid condition the creatinine picrate colour faded, and by

Heingard and Tiderstrom (1973) who further simplified the procedure by eliminating the need for protein precipitation. The colour was destroyed by acid pH. The difference in colour intensity, which was measured spectrophotometrically at 500 nm before and after acidification, was proportional to creatinine concentration.

3.2 Other experimental tumours studied

3.2.1 MAC13 colon adenocarcinoma

Fragments of the MAC13 adenocarcinoma, supplied by Dr J Double, Bradford University, were implanted in the flank of male NMRI mice. This tumour was found to have a doubling time of 7 days as determined by Mr Mike Wynter, Aston University. There was no weight loss experienced during the growth of the tumour.

3.2.2 MAC15A cell line

MAC15A cells were grown in tissue culture in RPMI 1640 medium under an atmosphere of 5% CO_2: 95% air. These tumour cells were derived from an ascites tumour grown in NMRI mice by Dr M. Bibby, Bradford University. The tumour did not induce weight loss in the animals. MAC15A cells were transplanted into mice from tissue culture to produce a tumour identical to the original.

3.2.3 Raji and GM892A cell lines

Raji and GM892A cell lines were obtained from Burkitts lymphoma and human lymphoblastoma respectively and were maintained by Dr M. Tisdale, University of Aston in RPMI 1640 medium containing 10% foetal calf serum. All cells were maintained under an atmosphere of 5% CO_2: 95% air and were passaged twice weekly.

Cells were seeded at an initial density of $5x10^4$ cells/ml and cell counts were determined by Dr M. Tisdale using a coulter electronic particle counter.

3.3 MAC16 cell line

The MAC16 tissue culture line was one of many cell lines derived by Drs J.A. Double and M. Bibby at the University of Bradford from the *in vivo* MAC16 tumour. It was the only cell line capable of producing cachexia when re-injected into NMRI mice. Cells were grown by Dr M. Tisdale, University of Aston, in RPMI 1640 culture medium with an atmosphere of 5% CO_2: 95% air. Doubling time of the cells was approximately 38 h.

3.4 In vitro determination of the presence of lipolytic factors in the MAC16 colon adenocarcinoma

The following two methods were employed to determine the presence of lipolytic factors:

3.4.1 Measurement of free fatty acid production

Male Balb/c mice were killed by cervical dislocation and their epididymal adipose tissue was quickly removed and minced in Krebs-Ringer bicarbonate buffer (pH 7.6). Approximately 50-100mg of the adipose tissue was incubated with homogenised tumour supernatant in a total volume of 0.25 ml of the Krebs-Ringer bicarbonate buffer. Controls containing adipose tissue and buffer alone were included in each experiment and the spontaneous release of free fatty acids was subtracted from the values obtained with tumour present. Incubations were continued for up to 2 h at 37°C and the FFA concentrations in the cell-free supernatants were determined using a Wako NEFA C kit (section 3.1.4).

3.4.2 Measurement of glycerol production

3.4.2.1 Preparation of isolated adipocytes

MF1, BKW or Balb/c mice were killed by cervical dislocation and their epididymal adipose tissue removed and placed in isotonic saline. The adipose tissue was then minced and incubated at 37°C for 2 h in Krebs-Ringer bicarbonate buffer containing collagenase (1.5 - 2.0 mg/ml). The cells were gassed prior to incubation with 95% O_2: 5% CO_2. Digestion of the tissues was detected by the disappearance of intact pieces and an increased turbidity of the medium. Undigested and non-adipose matter was removed by allowing the fat cells to float to the surface of the

buffer and the infranatant was aspirated, being replaced with fresh buffer. This washing procedure was repeated five times to remove all collagenase, non adipose cells and any endogenous hormones.

After washing the cells were suspended in an appropriate amount of buffer, stirred to uniformity using a magnetic stirrer, and aliquots taken for cell counting. The cells were counted using a Neubauer haemocytometer and the volume of buffer adjusted to give a cell density of $1-2x10^5$ adipocytes/ml.

3.4.2.2 Lipolysis assay

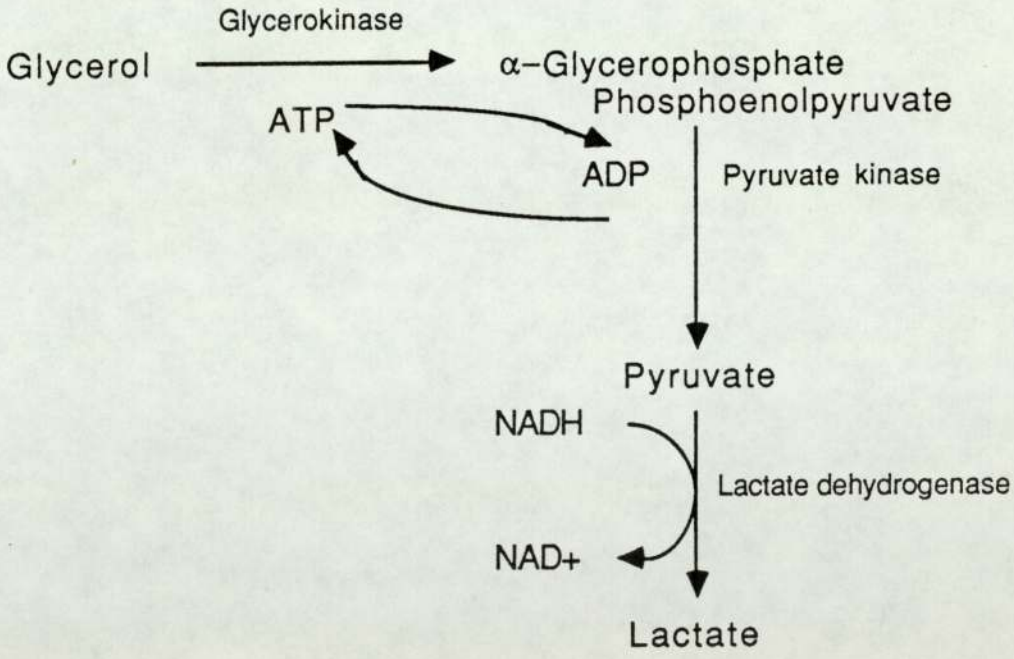
Cell samples (1.0 ml) were incubated with the appopriate test substance and gassed again with 95% O_2: 5% CO_2. The incubation was for 2 h at 37°C in a shaking water bath. Control samples containing adipocytes alone were also analysed to measure any spontaneous glycerol release. When assaying serum samples a control (no adipocytes) was also included to measure the initial amount of glycerol present in the serum. At the end of the incubation period 0.5 ml of the incubation buffer was added to 0.5 ml of perchloric acid (10% w/v) and the mixture was shaken to ensure deproteinisation. The precipitated protein was sedimented by centrifugation at 2,000 rpm for 10 min in a Heraeus labofuge 6000 centrifuge. The supernatant was aspirated using a Pasteur pipette. The supernatant was then neutralised with KOH (20% w/v) after which the volume was recorded and used to calculate the dilution factor. The

potassium perchlorate precipitate was sedimented by centrifugation (2,000 rpm for 10 min) and the supernatant was aspirated. Assays were then either performed immediately or after storage at -20°C for between 18-72 h. The concentration of glycerol was determined enzymatically by the method Wieland (1974).

3.4.2.3 Glycerol determination

The level of glycerol in the samples was determined as indicated in the scheme below:

Figure 12



The reaction buffer was made up of triethanolamine (100 mM), $MgSO^4$ (2.0 mM), phosphoenolpyruvate (0.4 mM), NADH (0.25

mM), ATP (1.2 mM), pyruvate kinase (1 U/ml) and lactic dehydrogenase (7 U/ml). In each cuvette 0.83 ml of reaction mixture was added plus 200 µl of sample. The blank contained 0.83ml of reaction mixture and 200 µl of distilled water. All samples were calibrated against distilled water alone (no buffer). The optical density was read at 340 nm for 2 min until it was stable and then 10 µl of glycerokinase (1mg/ml) was added to each cuvette. The absorbance measurement continued to be read for a further 25 min. The change in absorbance was proportional to the concentration of glycerol present.

$$\frac{\text{ABSORBANCE} \times \text{dilution factor}}{6.22(\text{NADH extinction coefficient})} = \mu\text{mol glycerol}/10^5 \text{ adipocytes}$$

3.4.3 Tumour slice experiments

3.4.3.1 Preparation of tumour slices

MAC16 and MAC13 tumours were excised from male donor NMRI animals and placed in ice cold isotonic saline. The tumours were sliced evenly and the weights of the tumour slices recorded. The slices were kept in ice cold Krebs-Ringer bicarbonate buffer prior to experimentation.

3.4.3.2 Tumour slice lipolytic activity

Each tumour was cut into 3 slices and put into 0.5 ml of Krebs-Ringer bicarbonate buffer. One slice was homogenised and lipolytic activity determined as described

in section 3.4.2. The other slices were incubated at 37°C for either 1 or 2 h. After incubating, the lipolytic activity in the buffer surrounding the slices was determined. The tumour slices were then homogenised and reincubated for 2 h with fresh adipocytes. At the end of the incubation the lipolytic activity was determined. The decrease in lipolytic activity in the slices that were incubated for 1 or 2 h was due to the release of some of the lipolytic factors into the surrounding buffer. Therefore:

lipolytic activity of initial slice homogenised

- lipolytic activity of slice incubated for 2 h

= amount of activity released into buffer

Control fat cells without any tumour slices were also incubated for 1 or 2h to take into account any spontaneous lipolytic activity.

3.4.4 Protein determination

Protein was determined by the method of Bradford (1976) using Bio-rad reagent. Dilutions of protein samples were made (1:200 to 1:1000) and 0.8 ml was mixed with 0.2 ml of dye reagent and allowed to stand for 5 min. The absorbance was read spectrophotometrically at 595nm. All samples were calibrated against a control containing distilled water in place of the protein sample. A standard curve was constructed using bovine serum albumin and used to determine protein concentrations.

3.5 In vitro determination of the presence of proteolytic factors in the MAC16 colon adenocarcinoma

3.5.1 Preparation of samples

Mice were killed by cervical dislocation and their diaphragms removed and placed in ice cold isotonic saline. The diaphragms were cut into two and blotted. The hemidiaphragms were weighed and placed in 0.75 ml of Krebs-Ringer bicarbonate buffer where they were gassed with 95% O_2: 5% CO_2 for 10 s. The hemidiaphragms were incubated for 30 min at 37°C. This pre-incubation period removed any proteolytic breakdown caused by the removal and dissection of the diaphragms. The hemidiaphragms were then transferred to clean microfuge tubes containing test substances and made up to 0.75ml with Krebs-Ringer bicarbonate buffer. These were gassed with 95% O_2: 5% CO_2 for 10 s and incubated at 37°C for 2 h. At the end of the incubation period 0.5 ml of the surrounding buffer was removed and added to 0.125 ml of 50% cold TCA. The tubes were mixed and then centrifuged in a Beckman microfuge. The clear supernatants were transferred to clean tubes and were neutralised with 1 N NaOH.

The hemidiaphragms were homogenised in 1.0 ml of 0.01 M phosphate buffer (pH 7.4). The homogenates were transferred to centrifuge tubes containing 0.5 ml of 50% ice cold TCA. The samples were mixed well and centrifuged. The clear supernatants were transferred to clean tubes and neutralised with 1 N NaOH. All neutralised samples were assayed for

amino acid concentration. Control samples containing hemidiaphragm alone were also analysed to measure spontaneous proteolytic breakdown.

3.5.2 Spectrophotometric determination of amino acid concentration

A standard curve (0-10 mM) was constructed for alanine using 200 µl samples. This was used as a measure of total amino acid concentration in the surrounding buffer. Ninhydrin reagent (1.0 ml) was added to 200 µl of each sample and mixed well. The tubes were placed in a water bath at 100°C for 20 min. After cooling the diluent (1:1 propanol:water, 5 ml) was then added to each tube and mixed on a whirlimixer. The absorbance of the solution was read at 570 nm and the concentration of amino acids present in the samples calculated from the standard curve.

3.5.3 Tumour slice experiments

This was the same as described in section 3.4.3 but proteolysis was measured from mouse diaphragms. XKI hypernephromas were also assayed. Control diaphragms without any tumour slices were also incubated for 1 and 2 h to take into account any spontaneous proteolytic activity.

3.6 Characterisation of catabolic factors

3.6.1 Dialysis

A 10% w/v homogenate of the MAC16 tumour was prepared in Krebs-Ringer bicarbonate buffer and dialysed against distilled water overnight. After dialysis both the contents of the tubing and beaker were assayed for lipolysis as described in section 3.4.2.

3.6.2 Heat treatment

MAC16 tumour homogenate (0.5 ml) or partially purified factor (50 µl) was heated in a boiling tube at 90°C for 10 min. The homogenate was centrifuged and the supernatant was assayed for the presence of the lipolytic and proteolytic factors.

3.6.3 Acid treatment

The MAC16 tumour homogenate (0.5 ml) was added to 100 µl of 1 N perchloric acid, mixed thoroughly and centrifuged for 10 min at 2000 rpm. The supernatant was transferred to a clean tube and was neutralised with 1 N KOH. The neutralised sample was recentrifuged to precipitate any $KClO_4$ formed. The supernatant was decanted into a clean tube and assayed for the presence of the lipolytic and proteolytic factors.

3.6.4 Periodate treatment

An aliquot (100 µl) of the partially purified factor was oxidised by treating it with 100µl of 0.1 M sodium periodate in sodium acetate buffer (pH 4.5) at 25°C for 16 h. The excess periodate was removed from the factor by passing it down a biogel P4 column and eluting with distilled water. Prior to the experiment periodate was passed down the biogel column of size 1.0 x 14.0 cm and the elution position was determined by adding KI. If periodate was present in the fraction then iodine woud be liberated shown by a brown discolouration. This showed that the periodate was clearly separated from the lipolytic factor on the biogel column.

3.6.5 RNAase/DNAase treatment

50µl of partially purified lipolytic factor was treated for 24 hr with 1.0 mg/ml of RNAase or DNAase at room temperature (20°C). Then 0.5ml was assayed for lipolytic activity using the glycerol assay as described in section 3.4.2 The activity measured was compared to the lipolytic activity of untreated factor.

3.6.6 Phosphatase/Sulphatase treatment

50µl of partially purified lipolytic factor was treated for 24 hr with 10 units/ml of alkaline phosphatase, acid phosphatase or sulphatase at 37°C in sterile

conditions. Then 0.5ml was assayed for lipolytic activity using the glycerol assay as described in section 3.4.2. The activity measured was compared to the lipolytic activity of untreated factor.

3.6.7 Effect of other compounds

The following compounds were assayed invitro for their effect on the catabolic activities of the MAC16 tumour:

	Conc.	Solvent
Propranolol	1.0 mM	Water
Indomethacin	1.0 mM	Water
RNAase and DNAase	1.0 mg/ml	Water
Insulin	10 U	Water
3-Hydroxybutyrate	8.0 mM	Water
Tumour necrosis factor	10^5 U	saline
Prostaglandins		
E_1, E_2, F_1 , F_2	5-20 µg/ml	Ethanol
Antitrypsin/ trypsin	1 mg/ml	Water
Phenylmethyl-sulphonylfluoride	0.5 mM	Water
Isobutylmethylxanthine	0.1/1.0 mM	Water

3.6.8 Guanidinobenzotase activity

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

3.6.8.1 Tumour preparation

MAC16 frozen tissue sections were prepared by immersing the solid tumour in isopentane and immediately quenching in liquid nitrogen. The frozen sections were prepared by L. Wilcox, University of Manchester.

3.6.8.2 Staining procedure

A flourescent probe, 9-aminoacridine, was used to stain guanidinobenzoatase. The staining and flourescent microscopy techniques were performed as described by Steven et al (1988) and were carried out at the University of Manchester.

3.7 Chromatographic techniques.

3.7.1 DEAE cellulose column chromatography

Supernatants from crude tumour homogenates were fractionated by anion exchange chromatography using a DEAE cellulose column eluting under a salt gradient. The DEAE cellulose column (dimensions 1.6 x 30.0 cm) was equilibriated with 0.01 M phosphate buffer (pH 8.0). The material was eluted with a linear gradient of 0 - 0.2 M NaCl in 0.01 M phosphate buffer (pH8.0). The column was eluted at a flow rate of 30 ml/h and the effluent from the column was collected in 5 ml fractions. A smaller DEAE column was also used (dimensions 1.0 x 14.0 cm) under the same

conditions as described. The column was eluted at a flow rate of 15 ml/h and the effluent from the column was collected in 1.0 ml fractions. Tumour protein (1.3 mg) was applied to the column. The lipolytic activity and the proteolytic activity of each fraction was measured by the lipolytic assay and proteolytic assay techniques described in sections 3.4.2 and 3.5.

3.7.2 Sephadex gel filtration exclusion chromatography

Fractions from the DEAE cellulose column that possessed significant lipolytic activity were concentrated by vacuum dialysis and the concentrate was applied to a Sephadex G150 column (size 1.6 x 30.0 cm). The column was equilibriated with 0.01 M phosphate buffer (pH8.0) and active material was eluted with the same buffer. The flow rate was 15 ml/h and the effluent was collected in 1.0 ml fractions. The lipolytic and proteolytic activity of each fraction was measured as described in sections 3.4.2 and 3.5.

The initial separation stage with the MAC16 tumour extracts used a Sephadex G150 column. In a further separation stage the active fractions from the DEAE cellulose chromatography were passed down a G50 Sephadex column to achieve a better resolution of the active peaks. A Sephadex G25 column was also used but it was not effective in retaining the active material. To determine an accurate molecular weight for the peaks the column used was calibrated with standards of known molecular weight.

3.7.3 Column chromatography of patients samples

Blood was removed from cancer patients and allowed to clot at room temperature for 10 min before centrifuging to collect the serum. The serum was stored at -20°C until use. Plasma, serum or urine (1.0 ml) was passed down a DEAE cellulose column as described in section 3.7.1. Active fractions from this column were subjected to Sephadex exclusion chromatography as described in section 3.7.2. Control serum samples from non-tumour-bearing individuals were also analysed for comparison. Plasma samples from Alzheimers patients were also assayed. Plasma samples from these patients were also combined (18 ml) and lyophilised and the concentrate was chromatograqphed on a Sephadex G50 column. Combined urine samples (128ml) from control subjects after 24 h starvation were also lyophilised and the concentrate subjected to Sephadex G50 chromatography. Urine samples (100 ml) from two cancer patients, one suffering from weight loss and one not, were lyophilised and the concentrate was passed down a Sephadex G50 column. All eluate from the Sephadex G50 column were then assayed for lipolytic activity as described in section 3.4.2.

3.7.4 Biogel column

Biogel columns were set up using Biogel P4 which was suitable for the separation of proteins of molecular weights between 2.0k and 4.0k daltons. The biogel was first soaked overnight in distilled water and then packed into a column

(size 1.0 x 14.0 cm). The lipolytic factor was applied and the column was eluted with distilled water at a flow rate of approximately 15 ml/ h. Fractions of 1.0 ml were collected. The biogel was used primarily to separate salts such as NaCl and periodate. The fractions were then analysed for lipolytic activity as described in section 3.4.2.

3.7.5 High Performance Liquid Chromatography (hplc)

Hplc was carried out at the Middlesex Hospital, London in collaboration with Dr B Coles, University of London. Various hydrophobic columns were used under the same conditions by using a gradient of acetonitrile: water/ trifluoroacetic acid (0.1%) run for 30 min from 10-60% acetonitrile. The UV absorbance was detected at 214 nm. A DE anion exchange column was also used with 0.01 M phosphate buffer (pH8.0) and a gradient of 0 - 0.2 M NaCl. The UV absorbance was detected at 214 nm. Fractions (0.4 ml) were collected and assayed at the University of Aston. Samples were concentrated to 100 µl on a gyrovap sample concentrator. Distilled water (0.4 ml) was added to each fraction and 100 µl of each fraction was assayed for lipolytic activity as described in section 3.4. The columns used were as follows:

C18 hydrophobic column

C8 hydrophobic column

C4 hydrophobic column

Amino column

Cyano column

DE anion exchange column

The best purification method was determined to be as follows:

1. Sephadex G150
2. Biogel P4
3. Sep-pak C18
4. Biogel P2 (University of London)
5. DE anion exchange (University of London)

3.8 Mechanism of action of lipolytic factor

3.8.1 Glucose consumption

MAC16 cells were grown in tissue culture in RPMI 1640 medium under an atmosphere of 5% CO_2: 95% air (as described in section 3.3). Glucose consumption was measured as described in section 3.1.3.

3.8.2 Lactate Production

Lactate was determined as follows:

3.8.2.1 Preparation of blood samples

Whole blood (50 µl) was deproteinised by the addition of 100µl of 1 N perchloric acid. The sample was mixed and

sedimented in a Beckman microfuge for 1 min. The clear supernatant was used for the determination of lactate.

3.8.2.2 Determination of lactate

Lactate was measured by the method of Gutmann and Wahlefield (1974). This method depends on the oxidation of lactate to pyruvate with the concominant formation of NADH which was determined from the increase in absorbance at 340 nm.

$$\text{L-(+)Lactate} + \text{NAD} \longrightarrow \text{Pyruvate} + \text{NADH} + \text{H}^+$$

Cuvettes were made up containing 0.43 M glycine - 0.34 M hydrazine hydrate buffer (pH9.0), 2.75 mM NAD and 50 µl of sample in a total volume of 3.0 ml. A blank was set up containing 1 N perchloric acid in place of the sample. All readings were calibrated against distilled water. The reaction was initiated by the addition of 20 µl of lactate dehydrogenase (19 units/ml) and the increase in absorbance due to the formation of NADH was measured at 340 nm for 30 min at 37°C.

3.8.3 Adenosine-3':5'-monophosphate (cAMP) analysis

Adipocytes were prepared as described in section 3.4.2.1. Adipocytes (1 - 2 x 10^5 cells) were incubated for the appropriate amount of time with the test substance or

substances at 37°C. The reaction was terminated by the addition of 1.0 ml of cold 5% trichloroacetic acid. This was rapidly equilibrated and centrifuged at 2,000 rpm for 10 min. The trichloroacetic acid was removed by extraction (5 times) with 4.0 ml of ether. The aqueous extract was lyophilised and dissolved in 200 µl of assay buffer (0.05M Tris pH7.5 containing 4mM EDTA). An aliquot (50 µl) of each sample was assayed for the concentration of cAMP present using a kit purchased from Amersham International, Bucks., UK(performed by Dr M.J Tisdale, Aston University).

The assay method was based on the competition between unlabelled cyclic AMP and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for cAMP. The amount of labelled protein-cAMP complex was inversly related to amount of unlabelled cAMP in the sample to be calculated. Separation of the protein bound cAMP from the unbound was achieved by adsorption of the free nucleotide onto coated charcoal, followed by centrifugation. An aliqout of the supernatant was then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample was then determined from a linear standard curve (Gilman , 1970 and Brown et al , 1971). The kit had a detection range between 0.2 - 16.0 pmoles of cAMP per incubation tube.

The test substances studied were eicosapentaenoic acid, salbutamol, adrenocorticotrophic hormone, tumour necrosis factor, prostaglandin E_1 and both the lipolytic and proteolytic factors.

3.9 Inducing cachexia in non-tumour bearing mice

Female NMRI mice were used in these experiments as they are smaller in body weight and therefore weight loss was more enhanced. Four groups of four mice were set up as described below:

Group 1: 100µl i.p with 0.9% saline

Group 2: 100µl i.p with MAC13 tumour homogenate

Group 3: 100µl i.p with MAC16 tumour homogenate

Group 4: 200µl i.p with MAC16 tumour homogenate

All injections were carried out between 9.30 - 10.30 am. The mice were injected for 5 days during which body weight and food and water intake were measured daily.

3.10 Presence of lipolytic factor in starvation

NMRI male mice, 10 - 12 weeks old, were starved for 24 h in metabolic cages. Weight loss was recorded. Urine and body composition analysis and plasma metabolite levels were determined as described in section 3.1.

Six human control subjects volunteered to starve for 24 h. During the starvation period only water intake was allowed. All six provided control urine, and one provided a blood sample, both prior to starvation and at the end of the starvation period. All samples from animals and humans were assayed for the presence of lipolytic factors before and after 24 h starvation as described in section 3.4. Urine creatinine was measured in all samples to standardise the

assay, (lipolytic activity was expressed per mg of creatinine). Post-starvation plasma and urine samples were chromatographed on a Sephadex G50 column under the conditions described in section 3.7. The fractions were assayed for lipolytic activity and compared with the profiles of lipolytic activity obtained from body fluid samples of cancer patients.

3.11 Inhibitors of cachexia

The following inhibitors were tested *invitro* using lipolytic and proteolytic assays as described in sections 3.4.2 and 3.5:

Eicosapentaenoic acid
Docosahexaenoic acid
Palmitic acid
Sodium butyrate
Sodium 3-hydroxybutyrate
Insulin
Megace
Salbutamol

Insulin, medium chain triglycerides (MCT) and Megace were tested *in vivo* as follows:

3.11.1 Insulin treatment

Ten to twelve week old male NMRI mice weighing between

26-28g were transplanted with the MAC16 colon adenocarcinoma. They were given food and water ad libitum. Once the mice started to lose weight and the tumours were palpable, usually about day 14 after transplantation, the animals were administered daily injections of isophane insulin (20 U/ kg s.c.). Injections were performed between 9.30 - 10.30 am for seven days. Two control groups were set up. One group of MAC16 tumour-bearing mice were injected subcutaneously with 0.9% saline while a group of non-tumour bearing mice were injected with 20 U of insulin/ kg/ day for 7 days.

Five groups of MAC16 tumour-bearing mice were injected subcutaneously with 200 µl of the following compounds:

Group 1: 0.9% saline
Group 2: 15 u insulin/ kg/ day
Group 3: 20 u insulin/ kg/ day
Group 4: 20 u insulin/ kg/ day with 30 mM sodium D(-)-3-hydroxybutyrate in the drinking water.
Group 5: 30 mM sodium D(-)-3-hydroxybutyrate in the drinking water.

After 7 days urine was collected for 24 h. Blood was removed by cardiac puncture and the levels of plasma metabolites determined. The carcass and tumour weights were recorded and the carcasses stored at -20°C prior to body composition analysis.

3.11.2 Dietary products

3.11.2.1 MCT dietary studies

3.11.2.1.1 Animals used

The non tumour bearing and MAC16 tumour bearing mice used were all male and weighed between 26 - 28 g. The mice were fed on normal rat and mouse breeding diet and given water ad libitum until the tumours were palpable and the mice started to lose weight. At this point the diets were initiated.

3.11.2.1.2 Dietary modifications

Four different dietary groups were studied:

1. Normal pelleted rat and mouse breeding diet plus water (see Appendix 1).
2. 80% MCT diet plus water.
3. Rat and mouse breeding diet plus water containing 30 mM 3-hydroxybutyrate ad libitum.
4. 80% MCT plus water containing 30 mM 3-hydroxybutyrate ad libitum.

All diets were isocaloric and isonitrogenous. The 80% MCT diet derived 80% of its calorific value from MCT and consisted of the following:

ANALYSIS % ENERGY x FAT(calc)	80%
NFE(indicating carbohydrate content and calculated by the difference at constant moisture)	22.8
RAW MATERIALS INCLUSION RATE	
Soya (dehulled)	410.0 g
Limestone	9.4 g
Bentonite (inert filler)	75.0 g
Salt	6.7 g
Dicalcium phosphate	45.75
Methionine	2.1 g
Rodent 006 premix	21.4 g
Triglyceride emulsion	565.0 ml

	1135.0 g

Inclusion rates of raw materials are in g/ kg: energy 1244 kcal/ lb (11.48 mJ/ kg), protein 200 g/ kg. The triglyceride emulsion contained 52% MCT and 48% water and the following percentages of saturated fatty acids: C_6 1.1; C_8 81.1; C_{10} 15.7; C_{12} 2.1. The ketogenic diets were presented to the animals as a paste to minimise food scatter.

The normal rat and mouse breeding diet consisted of 50% carbohyrate and supplied 11.5% of the energy as fat (see Appendix 1).

During the experiment all mice were weighed and food and water intake was measured daily and food wastage determined. The dietary treatments were only continued for 8 days since by this time some of the control animals had lost 25% of their body weight and had to be exsanguated.

The urine and faeces was collected for nitrogen analysis and the following day blood was taken by cardiac puncture. Plasma metabolites were then measured as described in section 3.1. Carcass and tumour weights were recorded and the carcasses stored at -20°C prior to body composition analysis (section 3.1.7).

3.11.3 Megestrol acetate (Megace) treatment

Male NMRI mice (10 -12 weeks old) were transplanted with the MAC16 tumour. After approximately 14 days, when the animals were starting to lose weight and the tumours were palpable, they were injected subcutaneously with 3.0 mg of Megace/ day for 7 days or 3.0mg Megace/mouse twice a day for 3 days. Megace was made into a suspension using 100% pure corn oil (50 mg Megace in 3.0ml of corn oil). During the experiment all mice were weighed and food and water intake measured daily. 3.0mg Megace/mouse twice a day was only continued for 3 days due to the ocurrance of ulcerating tumours. At the end of the experiment the procedures described in section 3.10.1 were performed.

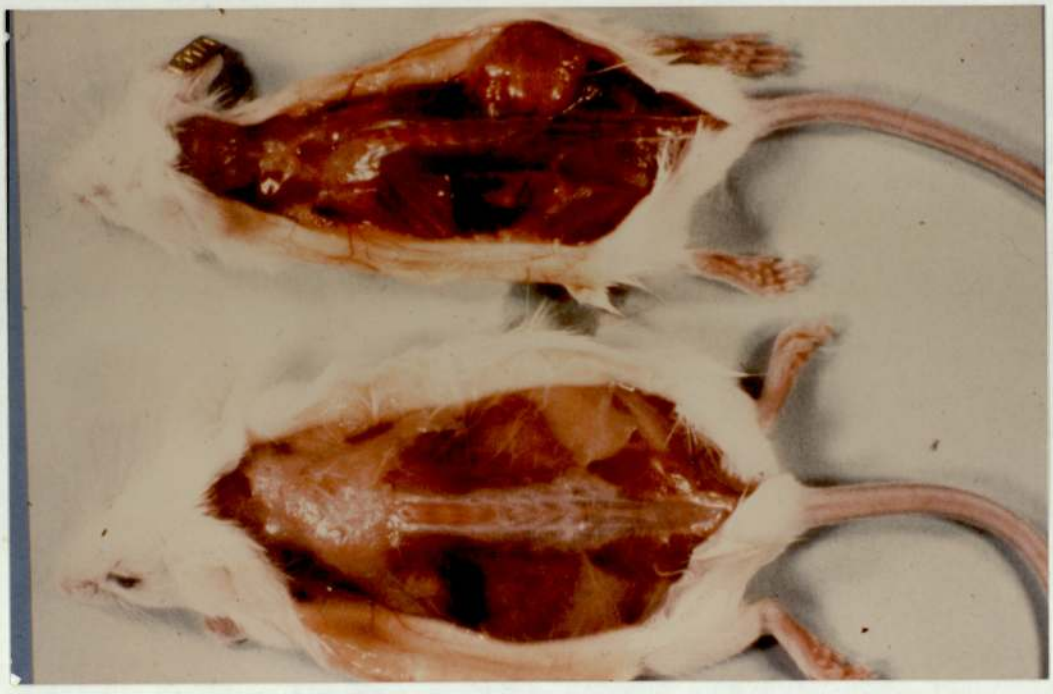
SECTION 4: RESULTS AND DISCUSSION

4.1 Characterisation of MAC16 colon adenocarcinoma in vivo.

4.1.1 Introduction

Cancer cachexia is characterised by a massive loss of body weight with extensive breakdown of both body fat and skeletal muscle. It is often, but not always, accompanied by anorexia (Costa, 1963). A significant weight loss is displayed by many patients with only a small primary tumour which may comprise often less than 0.01% of their total body weight (Nathanson and Hall, 1974). Animal models of cachexia to date have two disadvantages in relation to the human situation. 1)they possess a high tumour burden before weight loss becomes apparent and 2) a reduced food and water intake is required for weight loss (an anorectic response) (Morrison, 1973). As an experimental model we have studied a transplantable colon adenocarcinoma (MAC16) passaged in NMRI mice as a more suitable model of human cancer cachexia where weight loss occurs due to the metabolic effects of the tumour on the host. The weight loss produced with this tumour is seen as a reduction in the size of the animal and disappearance of fat deposits (Tisdale et al) (fig 13).

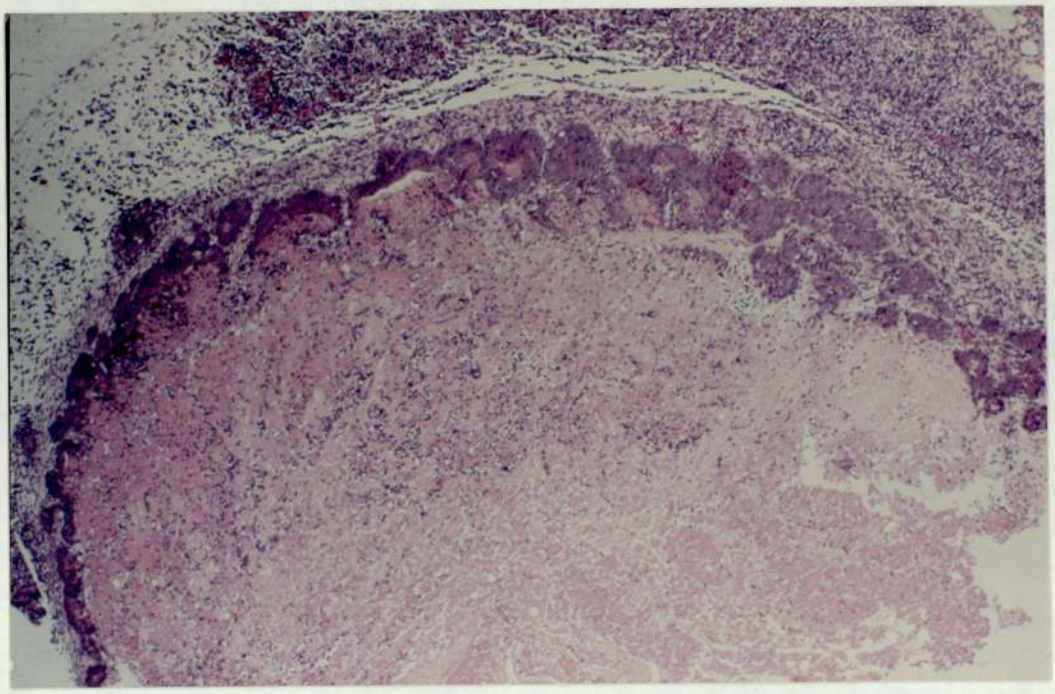
Figure 13



The effects of the MAC16 tumour on the growth of NMRI mice.
Both groups of mice consumed the same amount of food and water. The mouse on the right had a fragment of the tumour implanted 28 days prior to the photograph being taken.

The MAC16 tumour is a well differentiated colon adenocarcinoma induced with 1,2 dimethylhydrazine (Bibby et al, 1987a).
Histologically the MAC16 tumour is poorly vascularised with few blood vessels present and extensive necrosis at the centre of the tumour mass (fig 14).

Figure 14



Section through the MAC16 adenocarcinoma from mice fed the normal diet.

A negative nitrogen balance has been demonstrated by (Theologides 1972) in both animals and humans with a variety of tumours. Even when nitrogen intake was high enough to provide for both host and tumour, it has been shown that nitrogen was still lost from the carcass to the tumour (Mider et al 1948). Consequently nitrogen loss appears to be an important part of the cachectic syndrome and so urine analysis was studied to examine the nitrogen balance in the MAC16 tumour bearing animals.

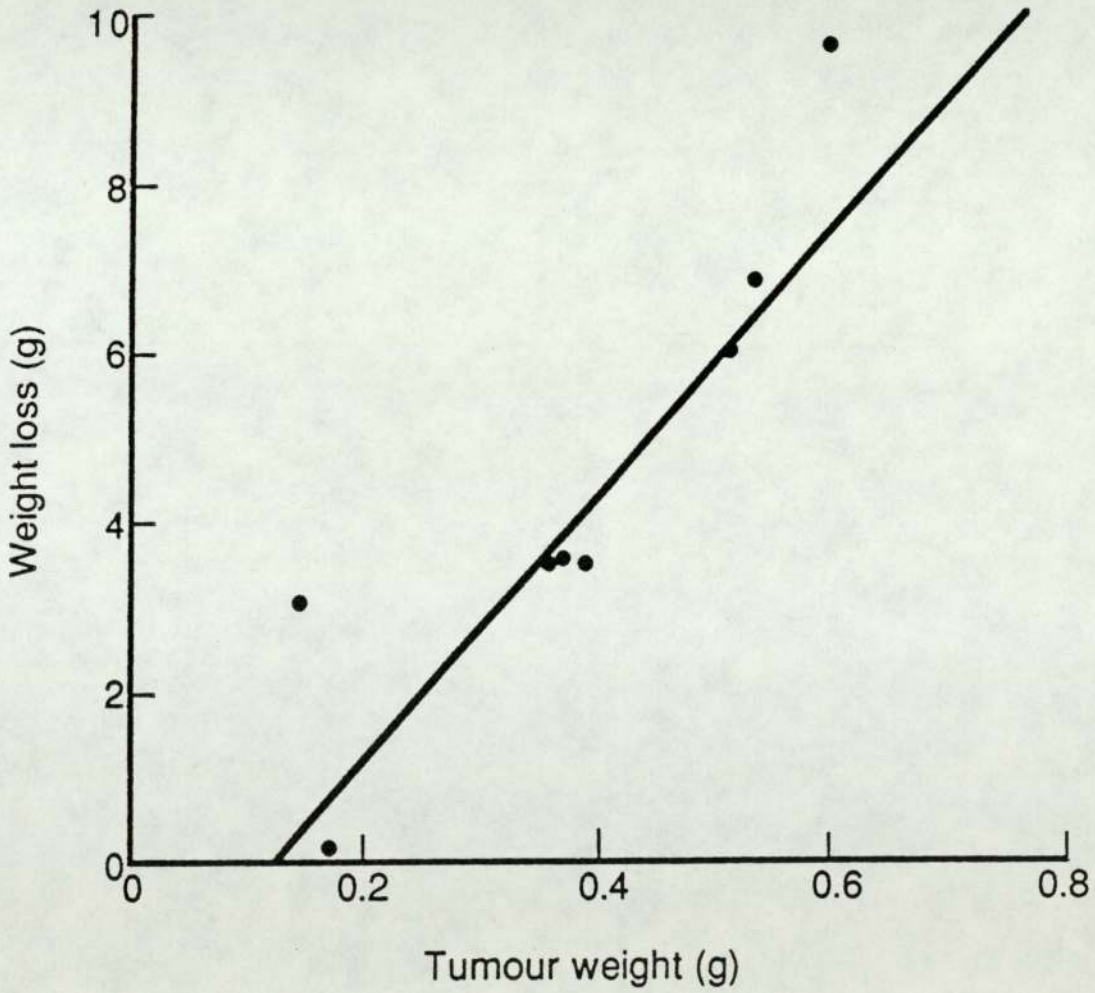
Weight loss was also observed in animals transplanted with a cell line derived from the solid MAC16 tumour by Dr M. Bibby, Bradford University.

4.1.2 Results

Animals transplanted with the MAC16 adenocarcinoma showed a progressive decrease in carcass weight as the tumour size increased (fig 15). The extent of weight loss experienced was directly proportional to the size of the tumour(fig 15) (correlation coefficient 0.91). The MAC16 tumour did not induce any anorectic response. The average food intake in tumour bearing animals (15.1±0.6 kcal/day) was not significantly different from that of non-tumour bearing animals (14.9±0.9 kcal/day), and both groups also had the same water intake (4.6±0.27 ml/day in tumour bearing animals compared to 4.8±0.16 ml/day in the controls). From the results in figure 15 it is apparent that the tumour must reach a minimum weight of 0.1g in a 30g male mouse before any weight loss occurs and the maximum allowable weight loss (33%) was observed with only a 0.6g tumour burden representing only 2% of body weight. The weight loss was associated with a decrease in both carcass fat and muscle dry weight. Both carcass fat and muscle dry weight also decreased in direct proportion to the weight of the tumour(correlation coefficient -0.93 fig 16 and 0.65 fig 17). This correlation between muscle dry weight and tumour weight did not reach the same significance as that between body fat and tumour weight possibly due to difficulties in dissection and weighing the thigh and gastrocnemius muscles accurately. Also since muscle degradation follows fat breakdown it was difficult to obtain points on the right hand side of the graph because of Home Office regulations,

Figure 15

The relationship between carcass weight (total body weight -tumour weight) produced by the MAC16 tumour in male NMRI mice and tumour weight.

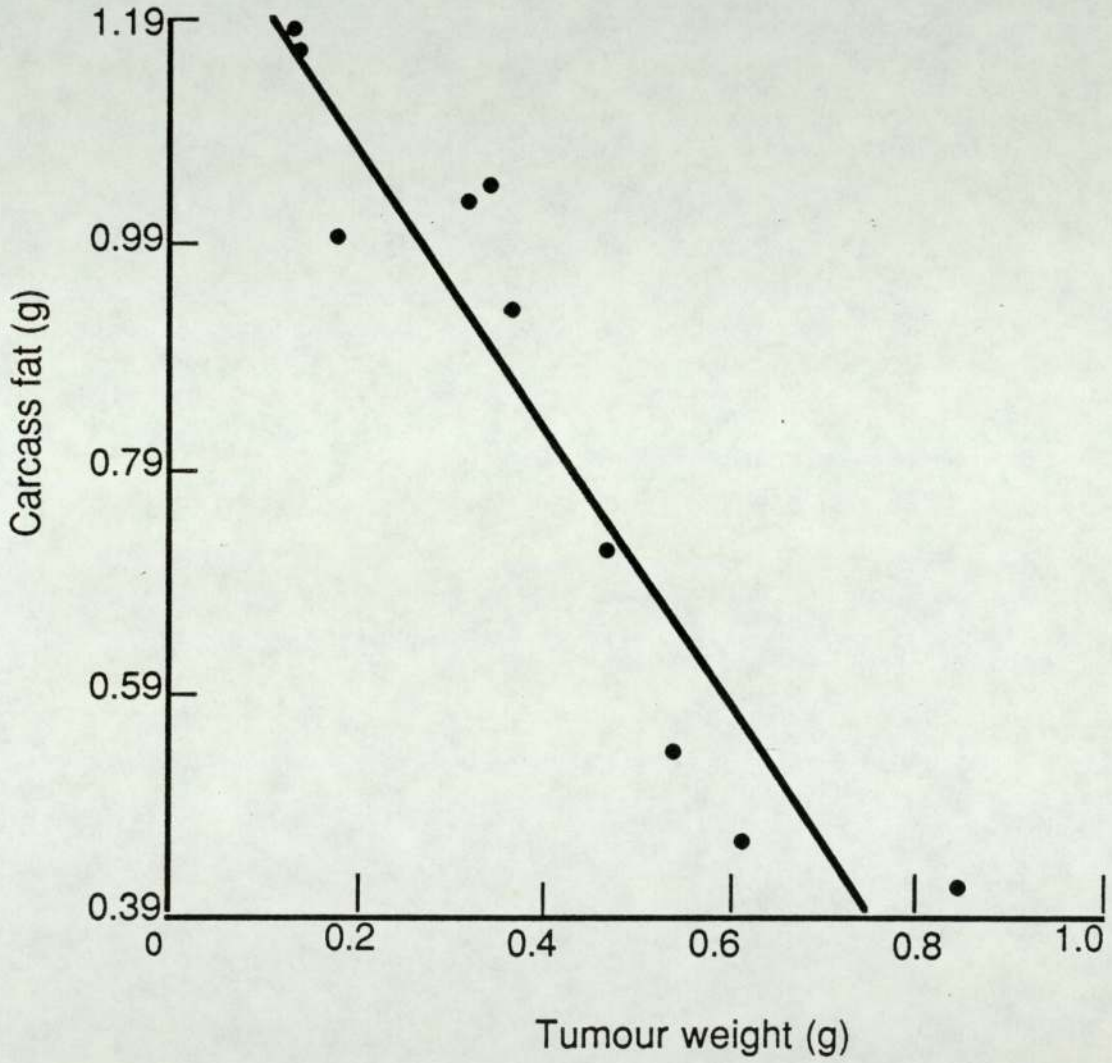


The results were fitted to a linear model by a least squares analysis ($r = 0.909$).

Each point represents the results from an individual animal.

Figure 16

The relationship between total body fat and weight of the MAC16 tumour in male NMRI mice.

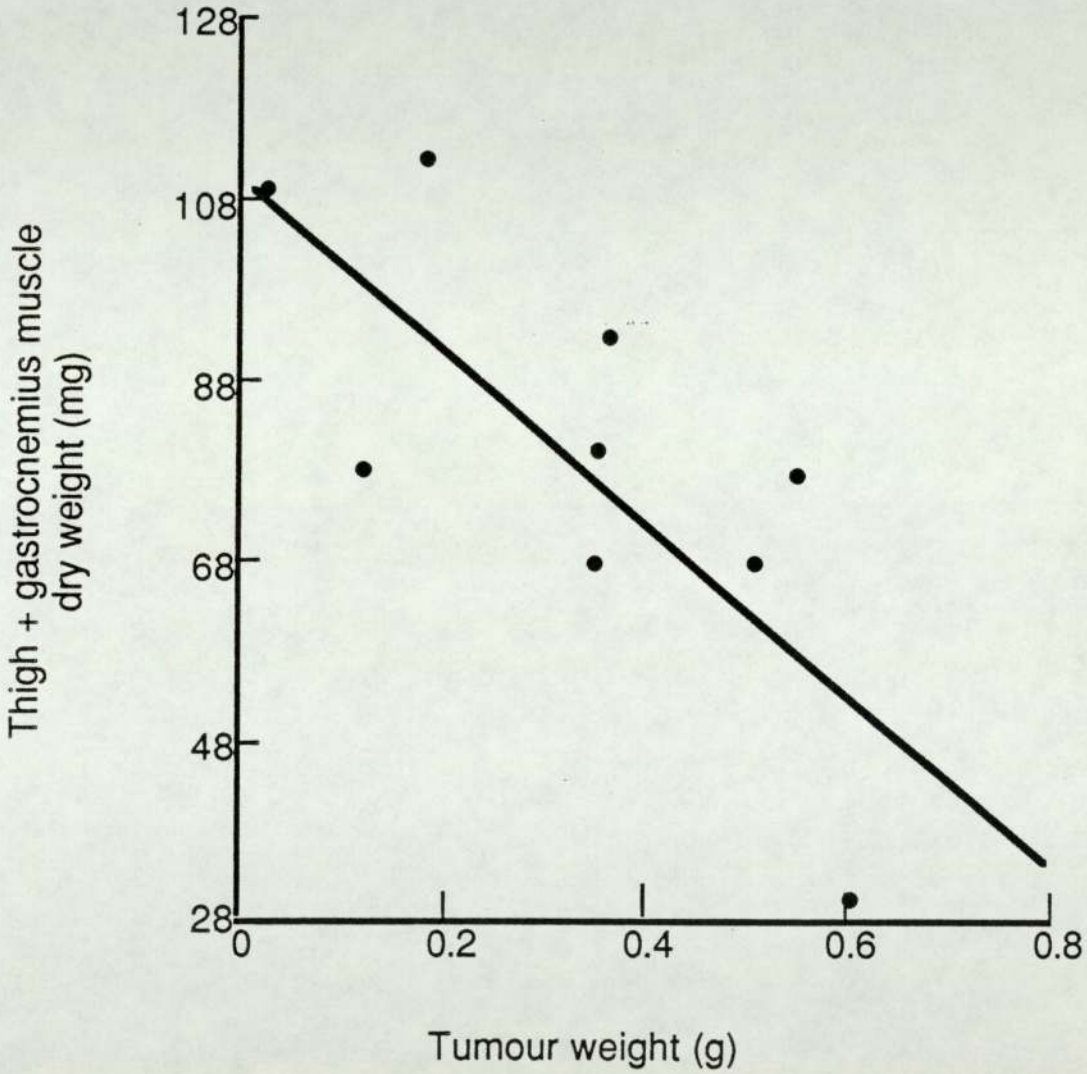


The results were fitted to a linear model by means of a least squares analysis (r=-0.93)

Each point represents results from an individual animal.

Figure 17

The relationship between thigh and gastrocnemius muscle dry weight and the weight of the MAC16 tumour in male NMRI mice.



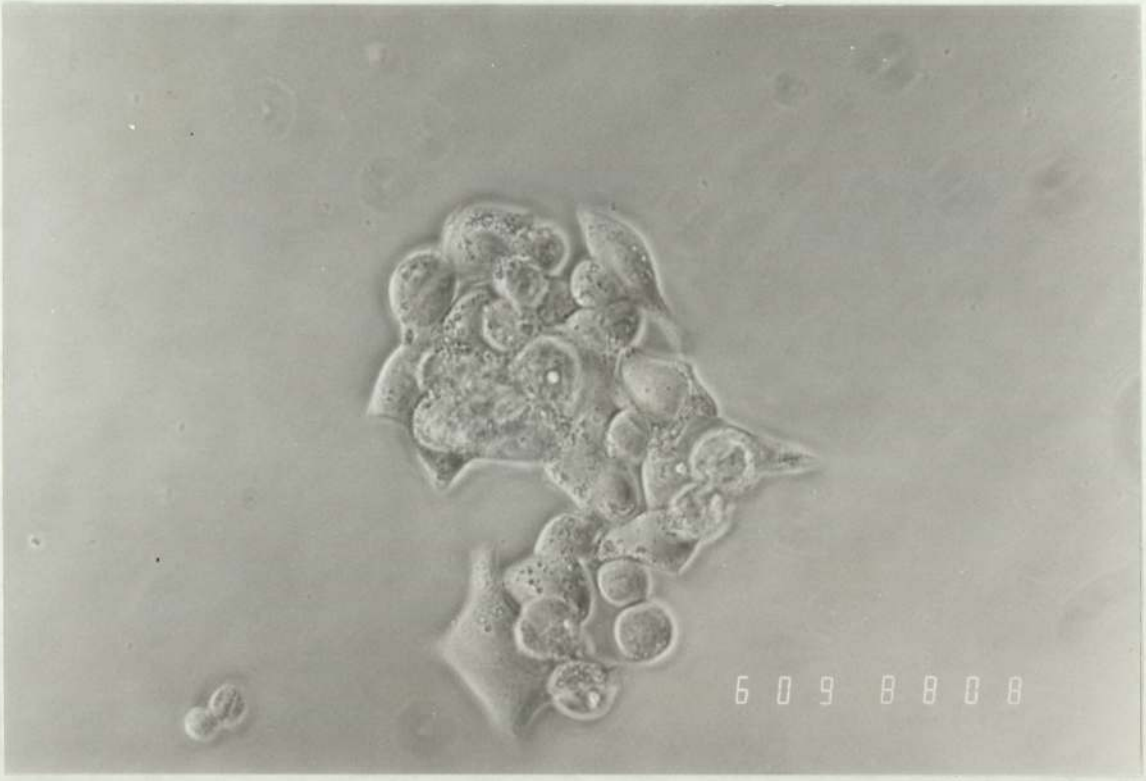
The results were fitted to a linear model by means of a least squares analys is (r= -0.651).

Each point represents the results from an individual animal.

which did not permit animals to lose more than 30% of their initial body weight. Loss of body fat exceeds that of muscle by about 13 times for a given weight of tumour. Total body weight decreased as the tumour weight increased and there was a corresponding reduction in the size of the individual body compartments (fig 19).

NMRI mice injected with the MAC16 cell line exhibited the same cachectic response as was seen in mice bearing the MAC16 tumour.

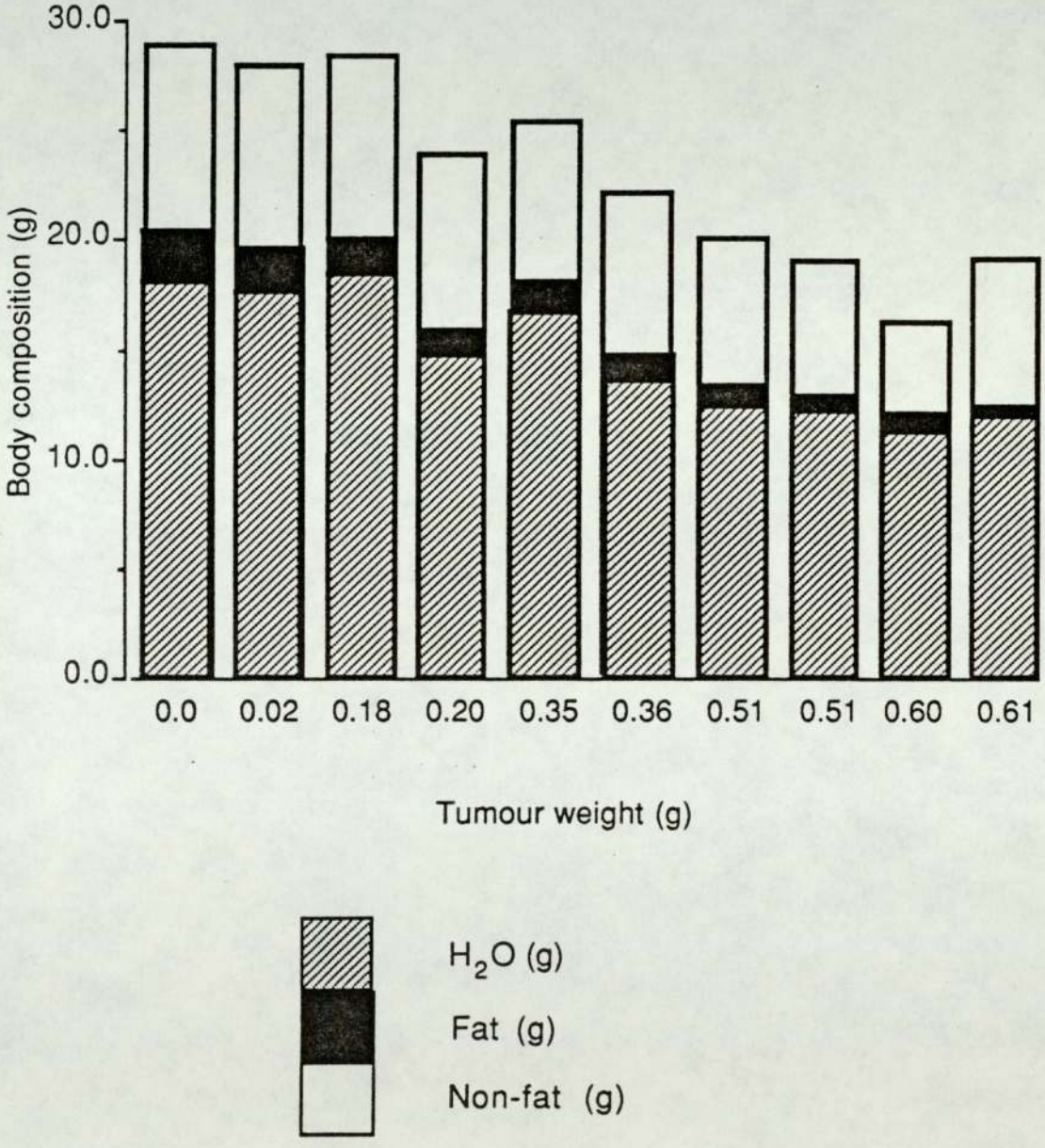
Figure 18



MAC16 cell line characterisitics

Figure 19

Body composition of male NMRI mice bearing the MAC16 tumour.



Each bar represents results from an individual animal.

2.6×10^6 cells were injected subcutaneously into the right flank of female NMRI mice. Weight loss was experienced after 12 days with no significant difference in food or water intake from non tumour bearing controls (table 1).

The MAC16 colon adenocarcinoma caused an alteration in plasma metabolites in the host when compared with control NMRI mice (table 2). The level of glucose in the blood was decreased in tumour bearing animals although the animals did not experience symptoms of hypoglycaemic shock. The MAC16 tumour also caused a significant decrease in the plasma levels of free fatty acids and triglycerides(table 2). There was no significant difference in the plasma ketone body levels between the tumour bearing animals and the non-tumour bearing animals.

The total urinary nitrogen excretion in tumour bearing animals varied according to the extent of weight loss. It was significantly elevated at small tumour burdens with weight losses up to 3g (fig 20). However above a 3-4g weight loss there was a conservation of nitrogen until at large weight losses the excretion level fell to or even below that found in non-tumour bearing animals.

The MAC13 adenocarcinoma is structurally related to the MAC16 but does not cause cachexia in its host. The MAC13 tumour was transplanted in male NMRI of weight 26-28g. Animals bearing the MAC13 tumour showed no elevation in overall nitrogen excretion and this did not change during the course of the study as was also observed in control non tumour bearing animals (table 3). The total nitrogen excretion of control non-tumour bearing animals and MAC13

Table 1

Cachectic activity of <u>in vitro</u> MAC16 cell line in female NMRI mice.

	weightloss(g)	food intake(g)	water intake(ml)
control	0.00±0.00	4.50±0.50	4.00±0.20
MAC16 cells	4.82±1.14	3.60±1.70	3.70±0.20

2.6×10^6 MAC16 cells were transplanted into female NMRI mice. Initial weight of 20g.

Weight loss was recorded 14 days after transplantation.

The results are expressed as the Mean ± S.E.M. The number of animals studied was 6 to 10.

Table 2

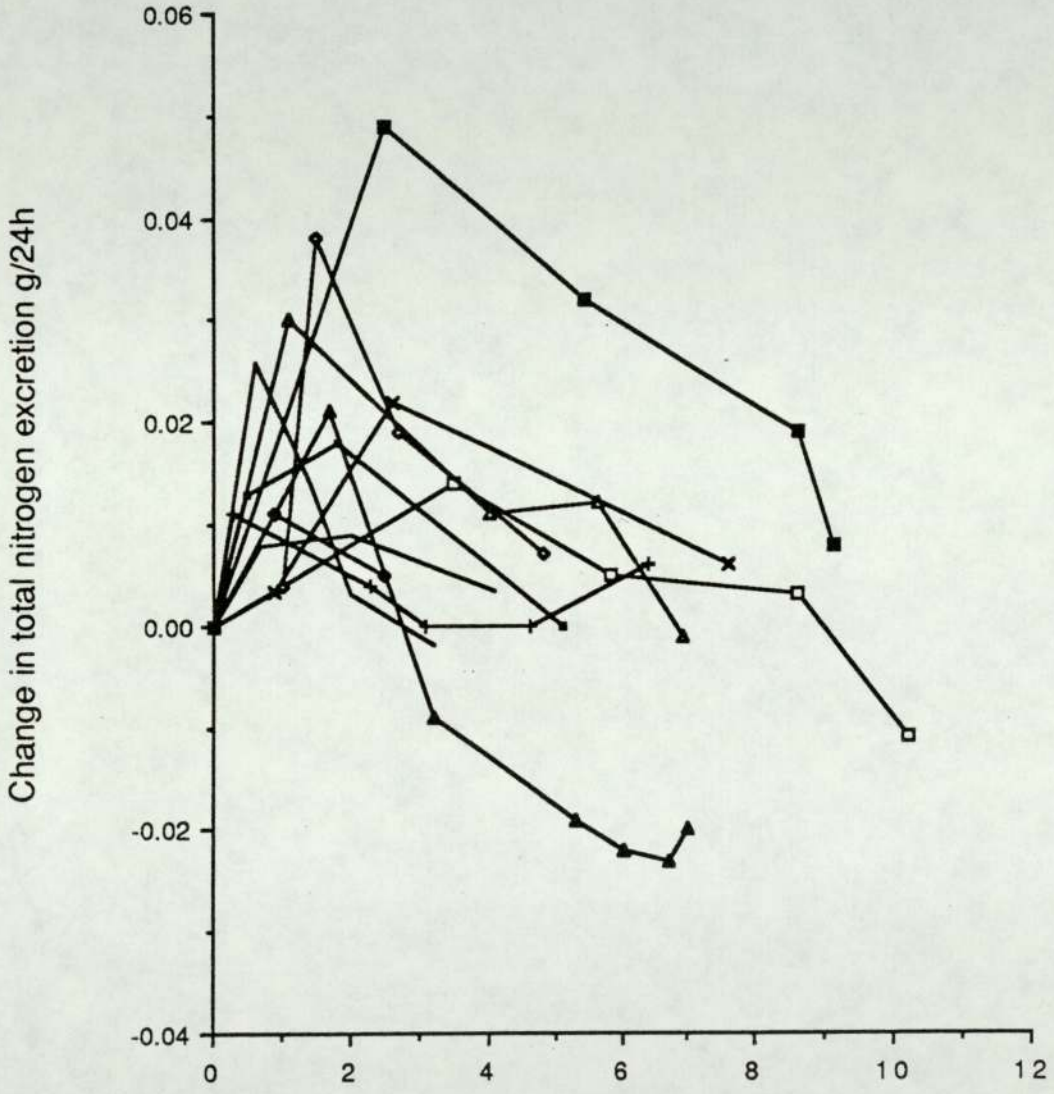
Plasma metabolites of MAC16 tumour bearing male mice

	Glucose mg/100ml	FFA mg/100ml	Triglycerides mM	Aceto acetate mM	3-hydroxy butyrate mM
Non-tumour bearing	136±5	29±2	1.15±0.11	0.040±0.002	0.078±0.007
MAC16 tumour bearing	108±11*	10±1**	0.50±0.07***	0.039±0.002	0.094±0.015

Results are expressed as the Mean ± S.E.M. The number of experiments performed were 6 to 8 . Significant difference *$p<0.05$, **$p<0.005$, ***$p<0.001$ compared to non-tumour bearing controls.

Figure 20

Total nitrogen excretion of male NMRI mice bearing the MAC16 tumour.



Each line represents results from an individual animal.

Total nitrogen excretion = urea+ammonia+creatinine.

Table 3

Effect of tumour type on weight loss and nitrogen excretion.

Tumour type	weight loss (g)	tumour wt (g)	totalnitrogen outputg/24hr	nitrogen balance g/24hr
none	0.00±0.00	-	0.030±0.003*	0.016±0.007**
MAC13	0.00±0.00	0.70±0.06	0.021±0.004**	0.025±0.007**
MAC16	5.68±0.54	0.66±0.10	0.042±0.003	0.004±0.007

Tumour type	Urea g/24hr	Ammonia 10^{-4} g/24hr	Creatinine 10^{-4} g/24hr
none	0.028±0.003*	3.91±0.50	6.44±0.86
MAC13	0.015±0.001**	13.8±5.1	3.87±0.09
MAC16	0.041±0.003	4.15±0.47	8.66±1.15

The results are expressed as the Mean ±S.E.M. The number of animals studied was 7 to 9per group. Significant difference *$p<0.05$, **$p<0.001$ compared to MAC16 tumour bearing animals . The total nitrogen input was 0.048±0.007 over 24 hrs and did not vary between the individual groups

adenocarcinoma tumour bearing animals did not change with time throughout the study.

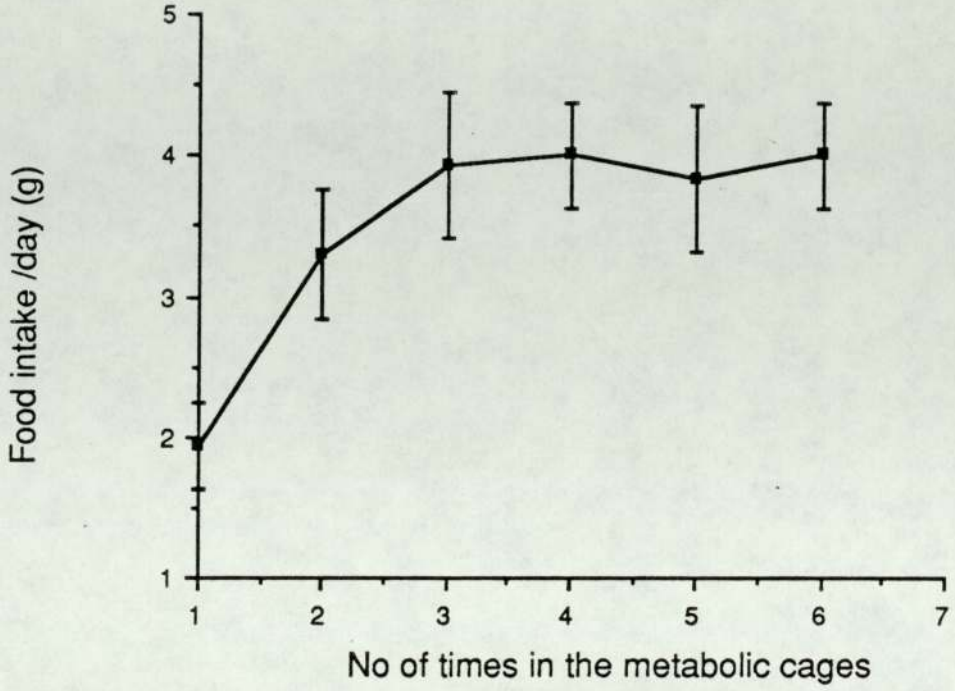
There was no significant difference seen in nitrogen excretion in the faeces between control and tumour bearing animals. Nitrogen intake was measured in order to be able to calculate the nitrogen balance of the animals. The nitrogen intake of the mice was significantly reduced during the first 24hr in the metabolic cages (fig 21). As the mice aclimatized to the cages nitrogen intake increased to a steady level as shown in (fig 21). Tumour bearing animals showed a negative nitrogen balance at small weight losses (fig 22) which recovered to or above the initial value at larger weight losses. Total nitrogen excretion equaled the addition of urea, ammonia and creatinine excretion. Urea excretion (fig 23) followed the same pattern with weight loss as did the total nitrogen excretion, while ammonia excretion overall did not change with weight loss (fig 24) and creatinine excretion decreased when large weight losses were experienced (fig 25) indicating a loss of muscle mass.

4.1.3 Discussion

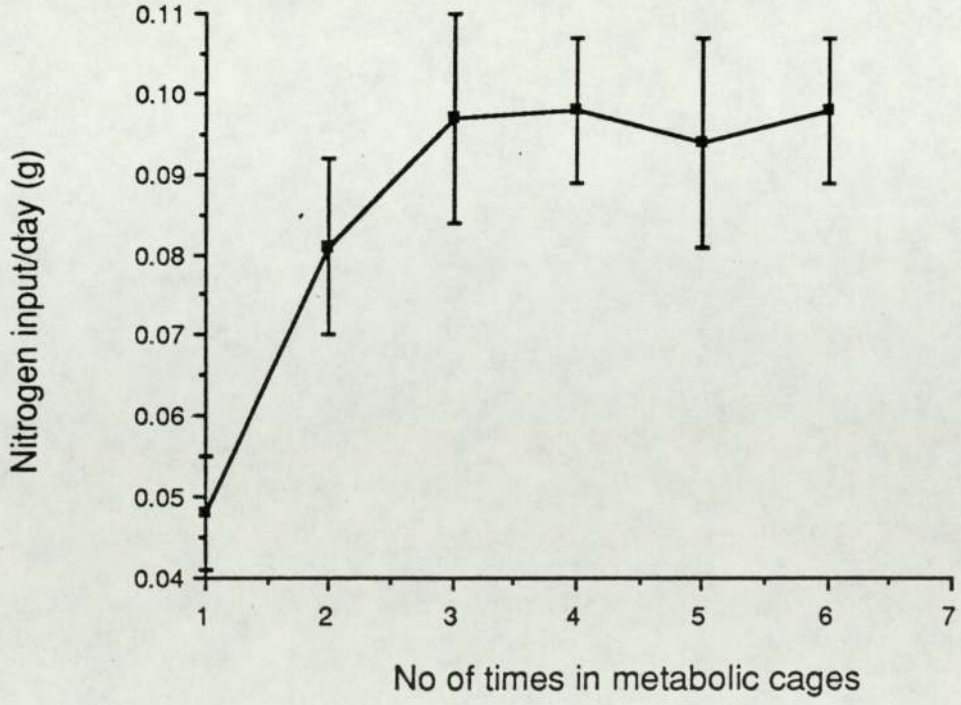
From these results it was concluded that the MAC16 tumour is a good model of human cachexia. Host weight loss occurs with a tumour burden >0.1g (0.3%) and reaches 30% with a 0.6g (2%) tumour burden and occurs without an effect on food and water intake. This situation is analogous to that in cancer patients where tumour burden rarely exceeds 5% of the total body weight. In addition some patients with

Figure 21

Food intake of male NMRI mice bearing the MAC16 tumour on consecutive days in the metabolic cages.



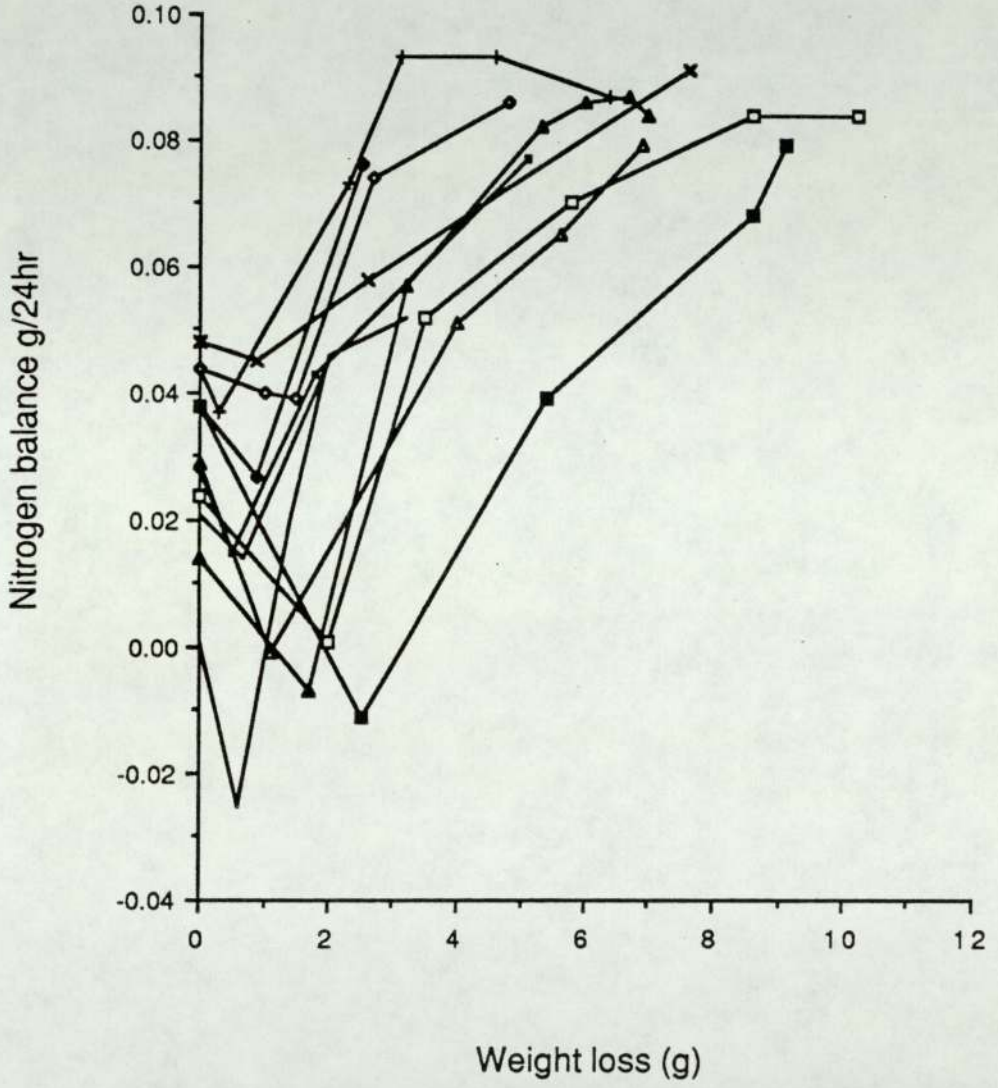
Nitrogen input of male NMRI mice bearing the MAC16 tumour on consecutive days in the metabolic cages.



The results are expressed as the Mean of 5 ± S.E.M.

Figure 22

Nitrogen balance of male NMRI mice bearing the MAC16 tumour.

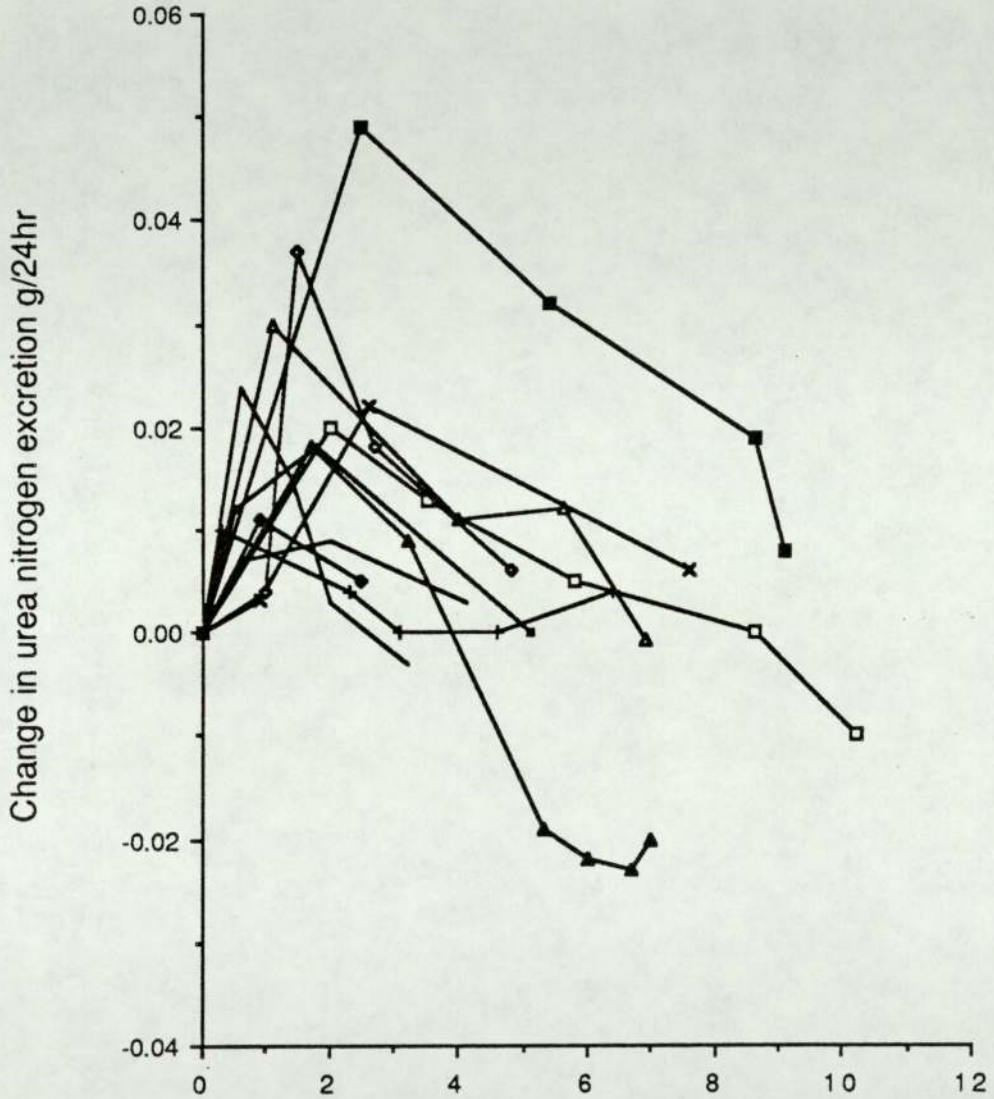


Each line represents results from an individual animal.

The nitrogen balance was calculated from nitrogen input -nitrogen output.

Figure 23

Urea excretion of male NMRI mice bearing the MAC16 tumour.

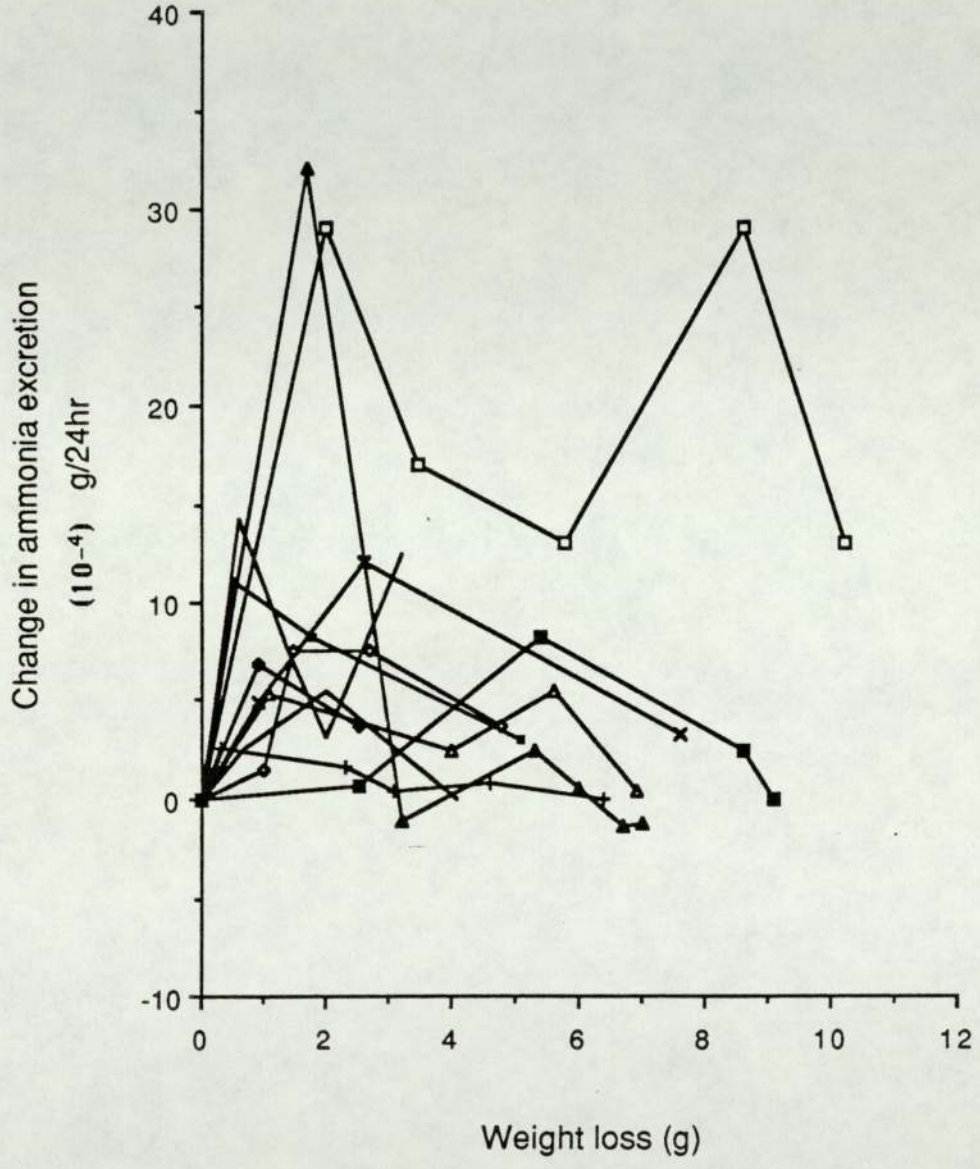


Each line represents results from an individual animal.

Urea excretion was measured in this animal when it had experienced different amounts of weight loss. Initial weights of the mice were 26-28g.

Figure 24

Ammonia excretion in male NMRI mice bearing the MAC16 tumour.

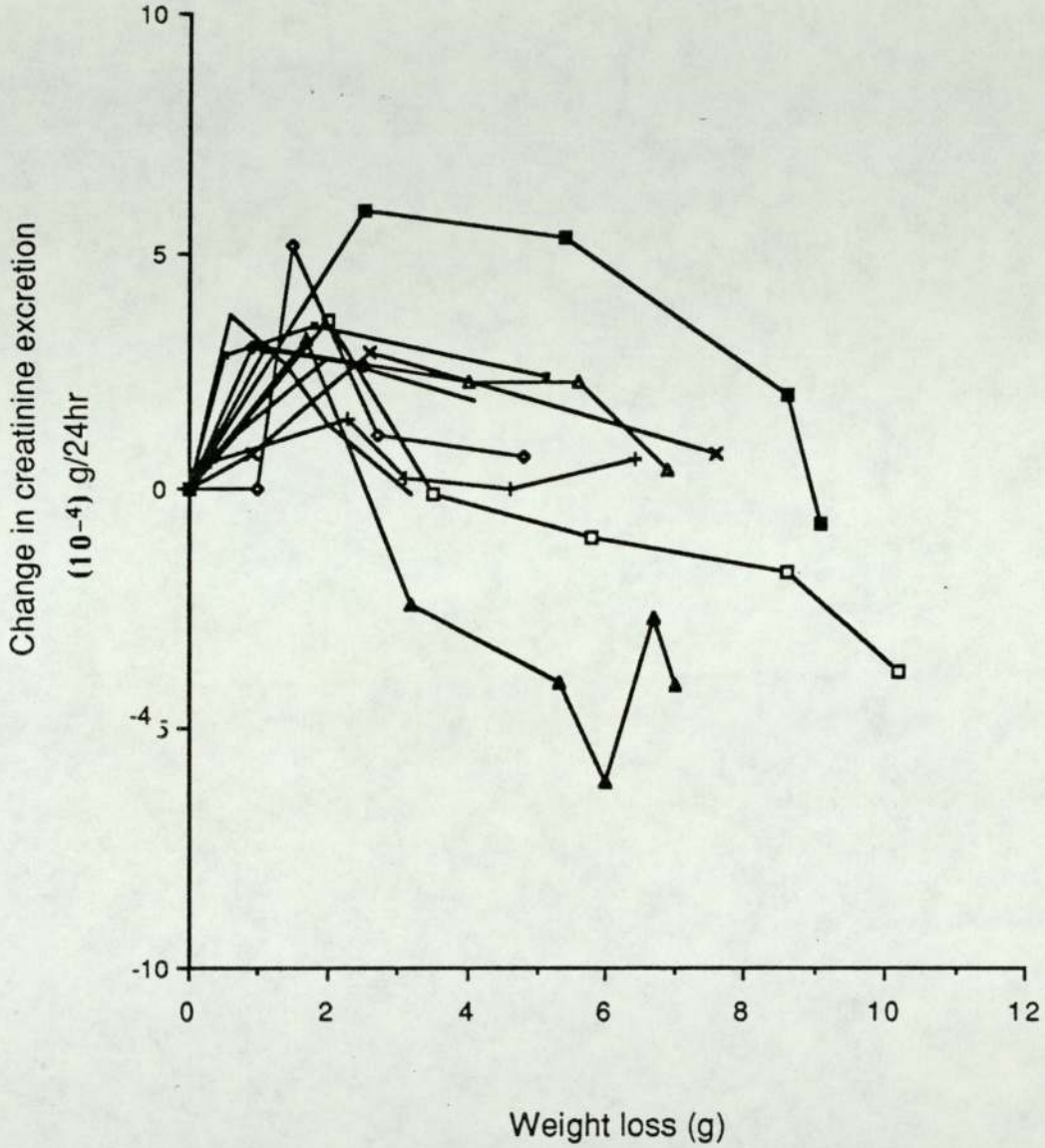


Each line represents results from an individual animal.

Ammonia excretion was measured in this animal when it had experienced different amounts of weight loss. Initial weights of the mice were 26-28g.

Figure 25

Creatinine excretion in male NMRI mice bearing the MAC16 tumour.



Each line represents results from an individual animal.

The creatinine excretion was measured in this animal when it had experienced different amounts of weight loss. Initial weights of the mice were 26-28g.

cancer cachexia do not have a reduced food or water intake again similar to our animal model.Most other experimental tumours have to reach 20 to 40% of the total body weight before symptoms of cachexia appear (Morrison, 1973). Strain et al (1980) have reported a human hypernephroma which produces > 25% weight loss in xenografted animals at tumour weights less than 5% of the total body weight. However some reduction of food intake was also reported.The tumour described here differs from that reported previously (Bibby et al 1987) in that weight loss occurs at much smaller tumour masses. This was due to the selection of a more cachectic tumour during transplantation.

The decrease in plasma metabolites in animals bearing the MAC16 tumour was most likely caused by the host and tumour competing for the available nutrients. The tumour may therefore be taking glucose, FFA, and triglycerides from the host to use for its own growth or it may stimulate the host to increase their metabolism. There was no increase in ketone bodies in the tumour bearing animals with weight loss indicating the absence of ketosis despite the large breakdown of fat deposits. Ketosis is normally induced during starvation(see introduction) to protect muscle protein from degradation by inhibiting the rate of oxidation of branched amino acids in muscle (Sherwin , 1975). The absence of ketosis in cancer patients (Conyers , 1979) and MAC16 tumour bearing animals therefore would allow gluconeogenesis from amino acids to continue. This subject is discussed further in section 4.7.

The elevated nitrogen excretion observed during the

progress of cancer cachexia was indicative of an increased mobilisation of body proteins for the production of glucose for use by the tumour. Alterations in host carbohydrate metabolism include an increase in glucose turnover (Lundholm et al ,1982),gluconeogenesis (Waterhouse et al 1974) and increased glucose recycling (Waterhouse et al 1979). The presence of a tumour alone was not sufficient to account for the increased nitrogen excretion seen, as animals with the MAC13 adenocarcinoma (of similar size to the MAC16 tumour), which did not develop weight loss have total nitrogen excretion values similar to non-tumour bearing animals. The elevated urea excretion in MAC16 tumour bearing animals was probably due to an increased gluconeogenesis from amino acids to provide glucose for the tumour, as the tumour in vitro has been shown to have an elevated glucose consumption (Tisdale et al, 1986). In the cachectic animals there appears to be a compensatory mechanism to prevent nitrogen excretion when the weight loss exceeds 3 to 4g in male mice as the nitrogen excretion was only elevated at small weight losses.

Cachexia was also displayed in animals bearing the MAC16 cell line derived from the in vivo tumour. Out of a series of cell lines derived from the solid tumour it was the only one which when reinjected into NMRI mice caused symptoms of cachexia. Therefore this shows that the cachexia exhibited was due to a tumour product and not a macrophage product like TNF and that the original tumour was heterogenous with regard to the cachectic response. However there are some reports of TNF production by tumour cells

(Oliff, 1987) and thus TNF levels were measured in animals bearing the MAC16 tumour. TNF was not detected in the MAC16 tumour or in the serum of tumour bearing animals with or without the effect of endotoxin(Mahony, Beck and Tisdale 1988). In addition the tumour mass was so small that it was unlikely that the tumour was simply competing with the host for the available nutrients. Again this implies the production of catabolic factors by the tumour which cause the breakdown of host muscle and fat stores. Experiments to determine the production of catabolic factors by the MAC16 adenocarcinoma are described in the next section.

4.2 Identification and characterisation of catabolic factors produced by the MAC16 adenocarcinoma, other tumour models and in the circulation of tumour-bearing animals and cancer patients.

4.2.1 Introduction

Theologides hypothesised in 1972 that cancer cachexia was caused by unidentified humoral factors. He further proposed in 1978 that these factors might be peptides, oligonucleotides or other metabolites produced by the tumour that could be responsible for the metabolic changes seen in the cachectic host. Since then many such factors have been identified but have not yet been shown to be the cause of cancer cachexia. Previous work has shown evidence for circulating factors in cancer anorexia/cachexia. Norton et al (1985) showed that parabiotic transfer of cachectic factors was possible in a rat tumour model, suggesting that cachexia was mediated by factors in the circulation.

Beutler et al (1985) have suggested that cancer cachexia is caused by a host response to the tumour in an attempt by the host to kill the tumour. They suggested that cachectin/TNF, a macrophage product, might cause the complex metabolic changes that lead to cachexia. Cachectin/TNF is a polypeptide with a molecular weight of 17K daltons that inhibits the enzyme lipoprotein lipase in peripheral tissues. Lipoprotein lipase hydrolyses triglycerides to glycerol and free fatty acids . It is suggested that cachectin/TNF inhibits this activity leading to an accumulation of triglycerides, which the body is incapable

of storing (Price et al 1986). Therefore more adipose tissue is broken down to satisfy the bodies needs for free fatty acids, ultimately causing weight loss. It has also been demonstrated (Torti, 1985) that cachectin/TNF inhibits adipocyte gene expression in TA1 adipocytes inducing an in vitro cachectic state. From these observations it was hypothesised that cachectin/TNF was the mediator of cachexia in vivo. Rats given a twice daily sublethal injection of recombinant human TNF for 8 days (Tracey et al, 1988) have displayed symptoms of anorexia, weight loss, depletion of whole body protein and lipid stores and anaemia. Work performed in our laboratory has demonstrated that TNF causes an acute anorexic effect. This differs from the metabolic changes associated with cachexia (Mahony, Beck and Tisdale 1988). After 24 hr the mice become tolerant to the effects of the TNF and start to regain weight (Mahony and Tisdale 1988). This has also been shown by Stovroff (1988) who likewise concluded that TNF has an anorexic effect. Further experiments in our laboratory have shown that this anorexia can be totally reversed by force feeding the mice glucose or by rehydration of the animals (Mahony and Tisdale 1989). Oliff et al (1987) studied the continuous infusion of TNF, by innoculating animals with TNF producing tumour cells that constantly produce low levels of circulating TNF. He showed 87% of the animals treated in this way developed weight loss. However, this weight loss was again accompanied with a decrease in food intake. Ultimately cachectin/TNF seems to play a role in anorexia rather than in cancer cachexia which often occurs without an acompanying anorexia (Costa,

1963).

Weight loss has not been noted as a side effect in any of the clinical trials of TNF/cachectin (Chapman et al 1987, Sherman et al 1988, Socher et al 1988 and Waage et al 1986). This suggests that TNF does not cause cachexia in man. This may be due to pharmacokinetic parameters of drug administration. A clinical study in patients with advanced cancer, given twice weekly doses of TNF for 4 weeks showed no weight loss (Chapman et al 1987). Even when TNF was continuously infused for 5 days no weight loss was observed, but anorexia was noted as a side effect (Sherman et al 1988). In addition TNF was not detectable in the serum of patients with clinical cancer cachexia (Socher et al 1988). In another study Balkwill et al (1987) found TNF-like activity in 50% of a random sample of cancer patients. However another group (Waage et al 1986) was only able to identify TNF in the serum of septicaemic patients but not in cancer patients. Therefore elevated levels of TNF may be present in some cancer patients possibly due to the presence of unknown secondary infections but it does not seem to be involved in the etiology of cachexia in cancer patients.

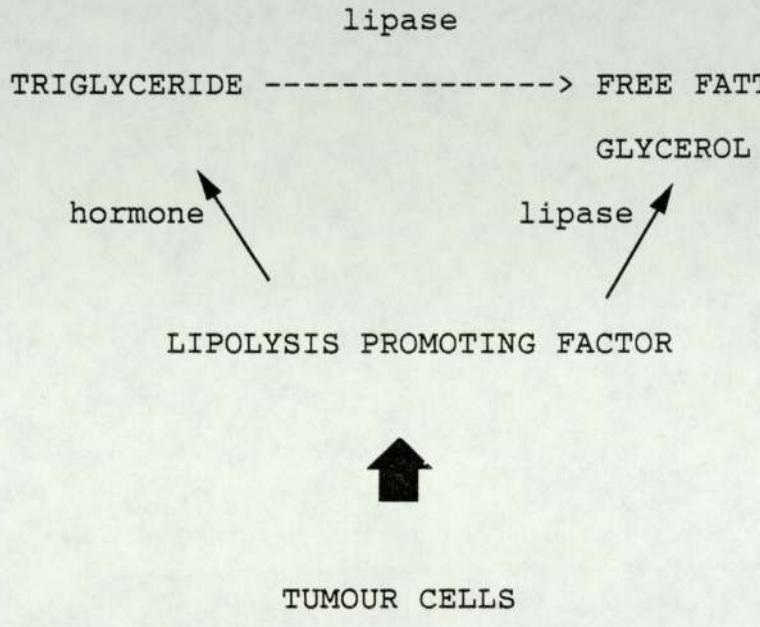
Alternatively, another view is that cancer cachexia may be caused by substances produced directly by the tumour which enter the circulation and have distant effects on the host. Many workers have advocated this idea. In 1947 Weil and Stetten showed the presence of a fat mobilising substance in the urine of fasting rabbits and Chalmers et al have shown a human lipolytic factor in fasting subjects (1958, 1960). More recently in 1980 Kitada et al reported

that the serum of AKR mice bearing a thymic lymphoma contained a lipid mobilising factor (LMF). They stated that this factor was necessary for the growth of the tumour, to derive the fatty acids required for membrane formation by mobilization from host tissues. The factor was also present in culture medium from an AKR lymphoma cell line (Kitada et al 1981). Kitada also showed that LMF activity was present in serum from a human patient with advanced cancer. In further experiments Kitada was able to identify the LMF as a small heat stable protein of molecular weight less than 10k daltons. LMF was shown to aggregate on standing in the cold before becoming active. LMF when injected back into normal mice produced a massive fat mobilization (Kitada et al 1982). This group has not published in this area since 1982 after stating that further characterisation of the LMF was in progress.

The presence of a lipolytic factor has also been reported by Masuno et al (1981). This lipolytic factor was found in the ascites fluid from DDK mice bearing sarcoma 180 and in the ascites fluid from patients with hepatoma. This factor, toxohormone-L, was a protein of molecular weight 75k daltons and had an isoelectric point of 4.7. Toxohormone-L was also heat labile and nondialysable. Further work by Masuno et al (1984) demonstrated that lateral ventricle injections of purified toxohormone-L into rats significantly suppressed food and water intake. Therefore, toxohormone-L was having either a direct anorexic effect or causing the production/release of an anorexigenic substance. This is a completely distinct lipolytic factor

from the one Kitada reported and discussed earlier.

The most recent report of a lipolytic factor was by Hollander et al (1987). They suggested from work on human melanoma cells, that certain tumour cell lines were capable of transferring a lipolysis-promoting activity to the media:



Another important characteristic of cancer cachexia is the wasting of skeletal muscle. This can be due to either a decrease in protein synthesis and/or an increase in muscle degradation (Rennie et al 1983). A decrease in in vivo protein synthesis has been shown in the muscle and liver of cachectic mice (Emery et al 1984). The possible production of tumour produced proteolytic factors has therefore been investigated.

It is well documented that increased proteolytic activity is associated with the presence of a cancerous growth. Malignant cells are known to possess many

proteolytic factors . These are involved in their loss of growth control, invasiveness and metastatic potential (Quigley et al, 1979). As early as 1925 Fisher showed that malignant tissue lysed plasma clots while normal tissue did not. Lysis of plasma clots is catalysed by proteolytic enzymes. In 1946 Fisher proposed that proteolytic factors were the cause of tumour invasion. Changes in peptidases and proteolytic activity in the plasma from tumour bearing animals have also been reported (Ottosen and Sylven 1960), suggesting the presence of circulatory proteolytic factors. Various human neoplasms have also been shown to produce collagenolytic enzymes. These would increase their ability to degrade connective tissue and help in the spread of metastases (Dresden et al 1972).

Recently Steven et al (1988) have identified a tumour -associated proteolytic enzyme, Guanidinobenzoatase. This enzyme is capable of degrading fibronectin. Guanidinobenzoatase is a tumour cell protease believed to be involved in cell migration. It has been identified in numerous tumours including the fluid of Ehrlich ascites tumour cells in mice. Inhibitors of this enzyme have also been identified in normal tissues and may explain why different tumours have different metastatic abilities. Greater knowledge of this enzyme and its inhibitors may ultimately help in the understanding of the regulation of metastasis (Steven and Griffin 1988). Other inhibitors, such as soya bean trypsin inhibitor and urokinase inhibitors when given repeatedly inhibit Ehrlich ascites tumour growth in vitro. This suggests another role for proteolytic

factors in the growth of the tumour (Verloes et al 1978).

In addition to cachexia muscle degradation is a characteristic in patients with trauma or sepsis. A plasma proteolytic factor has now been isolated from such patients and found to be a peptide of molecular weight of 4274 daltons (Clowes et al 1983). As yet no proteolytic factors have been identified in cachectic cancer patients.

The aim of the work in this section is (1) to identify any catabolic factors produced/released by the MAC16 adenocarcinoma and to further characterise the nature of these catabolic factors. (2) To identify the presence of these factors in other tumour models and/or circulating in the body fluids of rodents and humans.

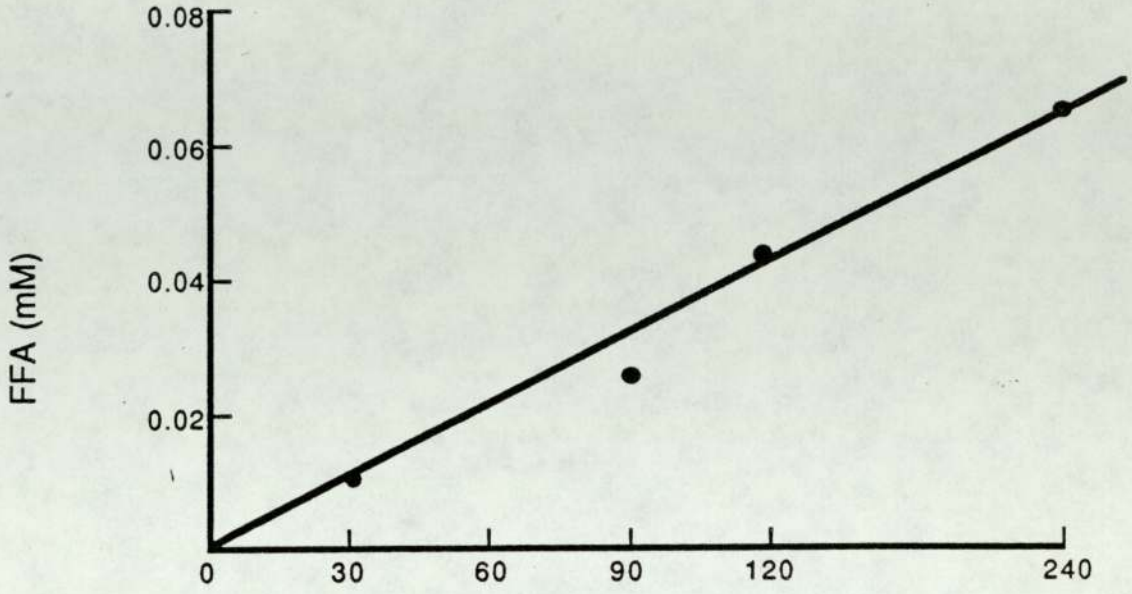
4.2.2 Results

4.2.2.1 Identification and characterisation of catabolic factors produced/released by the MAC16 adenocarcinoma.

In view of the extensive mobilization of body fat in animals bearing small body burdens of the MAC16 tumour the possible production of lipid mobilizing factors by the tumour has been investigated. Extracts of the MAC16 tumour caused an enhanced release of both free fatty acids and glycerol from fresh epididymal adipocytes, which increased with time upto 2 hr of incubation (fig 26). The MAC13 and MAC15A are structurally related adenocarcinomas neither of which produces symptoms of cachexia in NMRI mice. Cell extracts of these tumours also produced lipolysis but at levels much lower than those produced by the MAC16 tumour

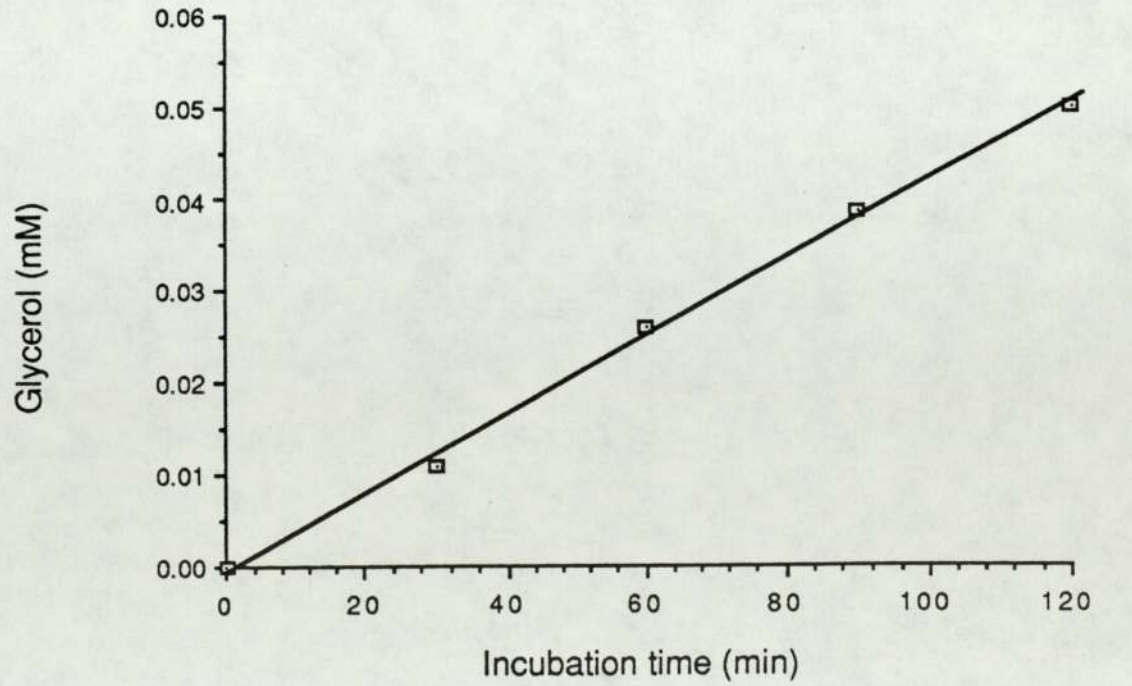
Figure 26

Rate of production of FFA from mouse fat pads by MAC16 tumour homogenate.



Tumour volume added per assay = 100µl

Rate of production of glycerol from adipocytes by MAC16 tumour homogenate.



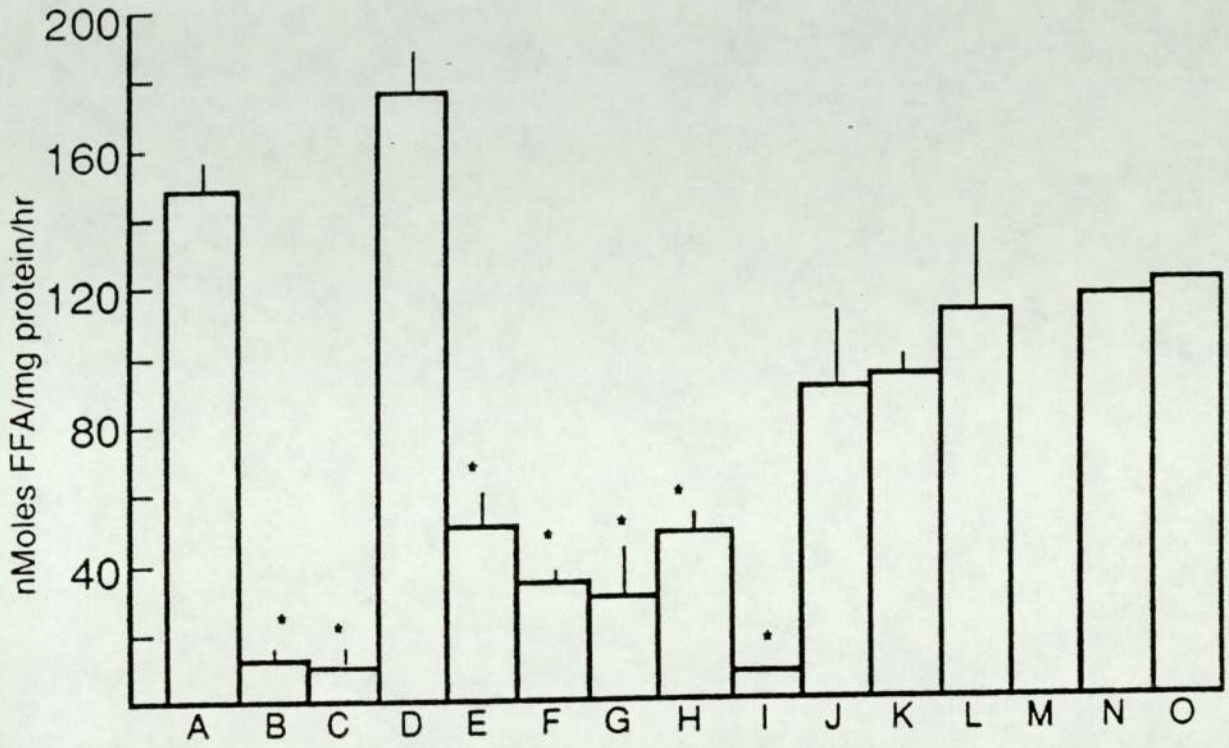
Tumour volume added per assay = 250µl

Protein content of tumour = 2.6 mg/ml.

(fig 27). To show the release of these factors tumour slice experiments were undertaken and demonstrated that the MAC tumours did release lipolytic factors into the surrounding medium (Table 4). It was noted that the MAC13 tumour contained 1/10th of the total activity of the MAC16 tumour but released more of its activity (72%) than the MAC16 tumour (51%). Similar to Rofe et al (1987) we have demonstrated that TNF has no direct lipolytic activity. Experiments were then carried out to further characterise the lipolytic factor/factors produced by the MAC16 adenocarcinoma. The lipolytic activty produced by the MAC16 tumour when tested in fresh homogenates was nondialysable and destroyed by heat and acid. The activity was not significantly affected by propranalol nor indomethacin suggesting that it was not due to β adrenergic agonists or prostaglandin production. Inhibitors of proteolytic enzymes like trypsin inhibitor and phenylmethylsulphonylfluoride also did not reduce the lipolytic activity of the MAC16 homogenate. However the MAC16 tumour induced lipolysis was significantly reduced by both insulin and 3-hydroxybutyrate. These agents also seemed to act synergistically. There was no effect on lipolysis by NaCl (up to 15mM) suggesting that the effect seen by sodium 3-hydroxybutyrate was due to the 3-hydroxybutyrate and not to the sodium salt or to the alteration in ionic strength. A large decrease in total carcass fat is seen with low levels of lipolytic activity (fig 28). However when carcass fat decreases in the animal large amounts of lipolytic activity are required to completely deplete the body of its fat stores and there

Figure 27

Rate of release of FFA from mouse epididymal adipose tissue by tumour homogenates.



A = MAC 16Tumour homogenate
B = MAC 16T HEATED TO 90°C
C = MAC 16T + ACID
D = MAC 16T DIALYSED
E = MAC 15A cell line
F = MAC 13 cell line
G = IOU INSULIN (I) + MAC 16T
H = 8.0mM 3 - HYDROXYBUTYRATE (3-HB) + MAC 16T
I = IOUI + 3 - HB(8.0mM) + MAC 16T
J = INDOMETHACIN 1mM + MAC 16T
K = PROPRANALOL 1mM + MAC 16T
L = 0.5mM Phenylmethylsulphonylfluoride + MAC 16T
M = 15.4mM NaCl
N = NaCl (15.4mM) + MAC 16T
O = TRYPSIN INHIBITOR(1mg/ml) + MAC 16T

The results are expressed as Mean ± S.E.M of 3 to 13 determinations. *indicates $p<0.001$ from A determined using the Students t test.

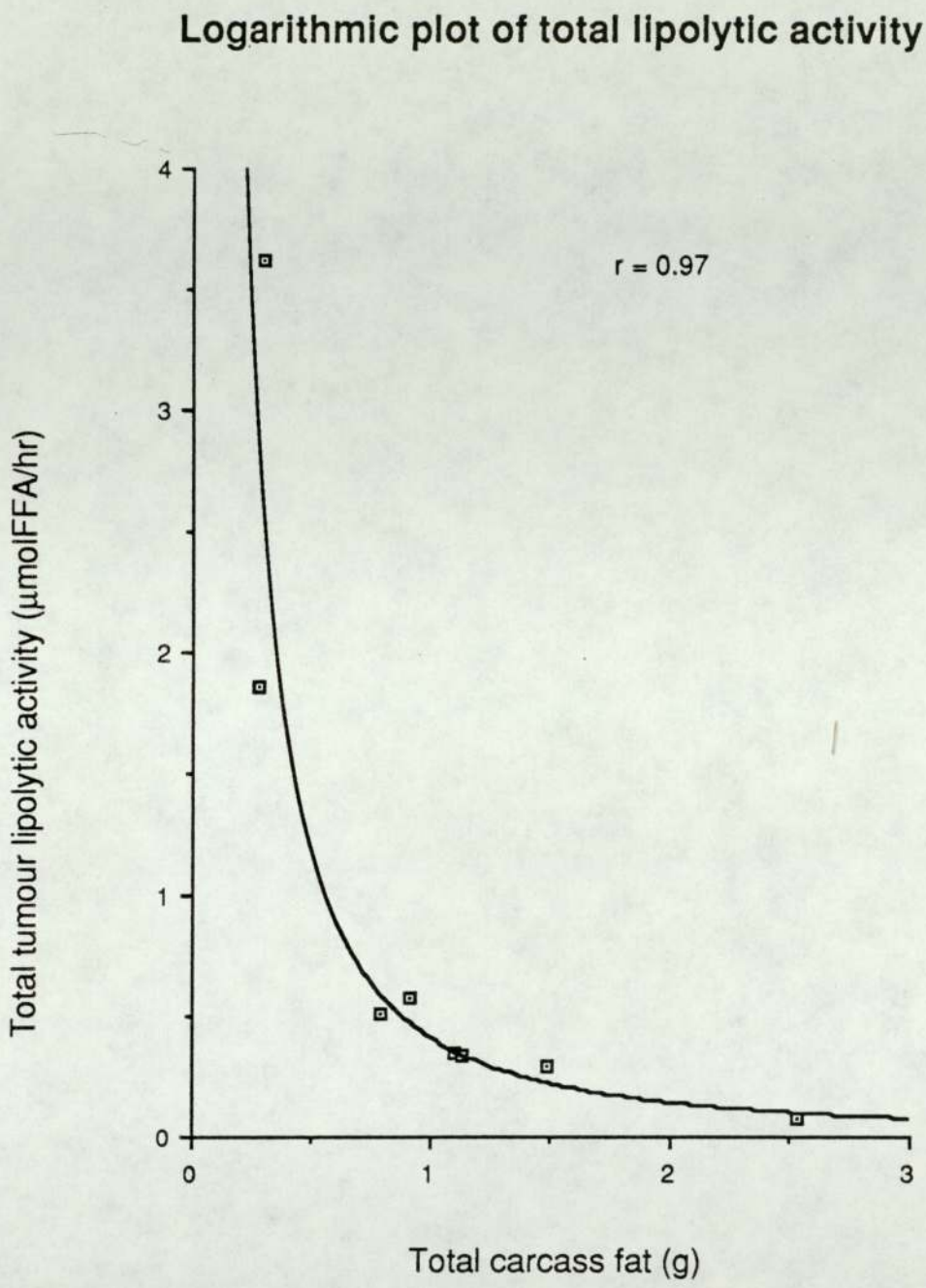
Table 4

Lipolytic activity of MAC16 adenocarcinoma slices.

sample	time of incubation(hr)	Lipolytic activity Glycerol(mM)/g		%tumour activity released
		medium	tumour slice	
MAC16	0	-	2.57	-
MAC16	1	2.27	1.75	32.0
MAC16	2	1.65	1.25	51.4
MAC13	0	-	0.287	-
MAC13	1	0.109	0.137	52.0
MAC13	2	0.074	0.080	72.1

The results are the Mean of two experiments.

Figure 28



Regression analysis of the relationship between

the total tumour content of lipolytic activity and total carcass fat.

appears to be a logarithmic relationship between the two. The lipolytic activity was not destroyed by either trypsin, chymotrypsin or proteinase K. but was substantially reduced by pronase (1mg/ml) a nonspecific protease. The lipolytic activity of the factor (16.69±2.15 mM glycerol) was significantly reduced by pronase (2.21±1.46 mM glycerol). This lipolytic factor/factors were also present in the plasma of MAC16 tumour bearing mice (table 5). This indicates that the MAC16 tumour releases a lipolytic factor(s) into the circulation and that this may be responsible for the extensive mobilization of body fat..

Further investigations were also undertaken into the production of proteolytic factors by the MAC16 adenocarcinoma, which could account for the decrease in carcass protein (fig 19). Extracts of the MAC16 tumour caused an enhanced release of amino acids when incubated with mouse diaphragms. This release was linearly proportional to incubation time up to 2 hr (fig 29). Unlike lipolytic activity neither the MAC13 and MAC15A cell lines produced any proteolytic factors (fig 30). The proteolytic factor/factors from the MAC16 tumour were shown to be released into the surrounding medium(table 6). Characterisation of these crude factors showed that they were destroyed by heat (90°C for 10 min), 1.0N perchloric acid, insulin (10U), 3-hydroxybutyrate (8mM) and phenylmethylsulphonylfluoride (0.5mM). The latter suggests that the proteolytic factor is a serine protease. Partial suppression of the proteolytic activity was shown by a trypsin inhibitor (1mg/ml). Neither RNAase, DNAase nor

Table 5

Plasma lipolytic/proteolytic activity in tumour bearing and non tumour bearing male NMRI mice.

Tumour type.	Lipolytic activity µmolFFA/hr/mlplasma	Proteolytic activity nmolAA/gD/ 2hr/mlplasma
MAC16 adenocarcinoma	1.29±0.04 *	15.50±4.41*
MAC13 adenocarcinoma	0.60±0.20	3.12±2.91
Non tumour bearing	0.63±0.05	0.00±0.00

Significant difference * $P<0.001$ compared to non tumour bearing

Table 6

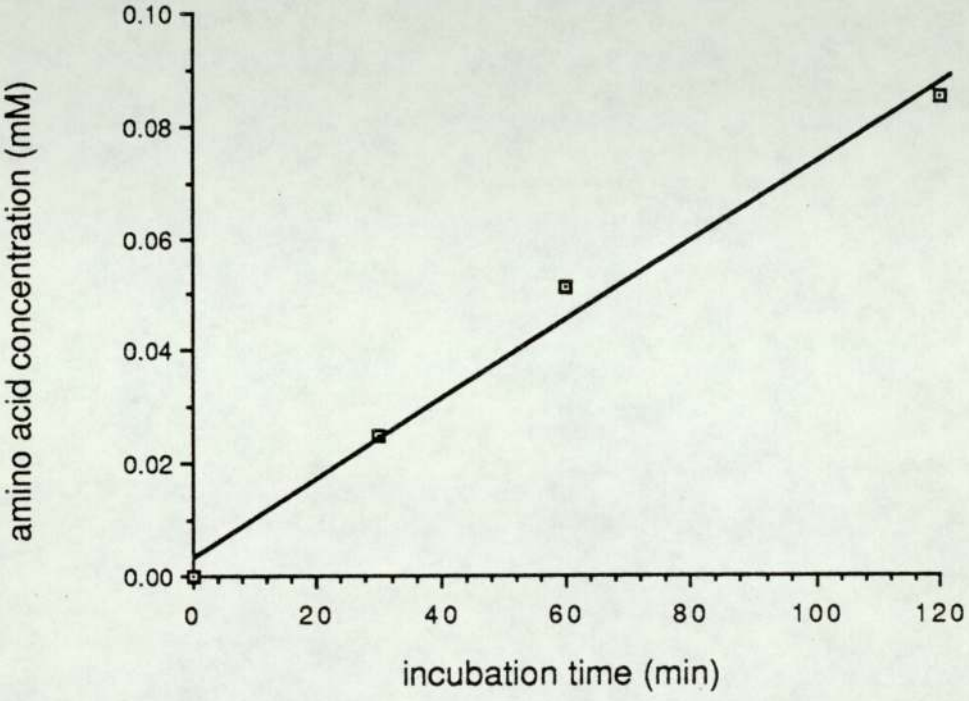
Proteolytic activity of MAC16 adenocarcinoma slices

sample	time of incubation(hr)	Proteolytic activity Aminoacid(mM)/gtumour		% tumour activity released
		medium	tumour slice	
MAC16	0	-	8.44	-
MAC16	1	9.22	5.49	35.0
MAC16	2	8.65	7.50	11.1

The results are expressed as the Mean of two experiments.

Figure 29

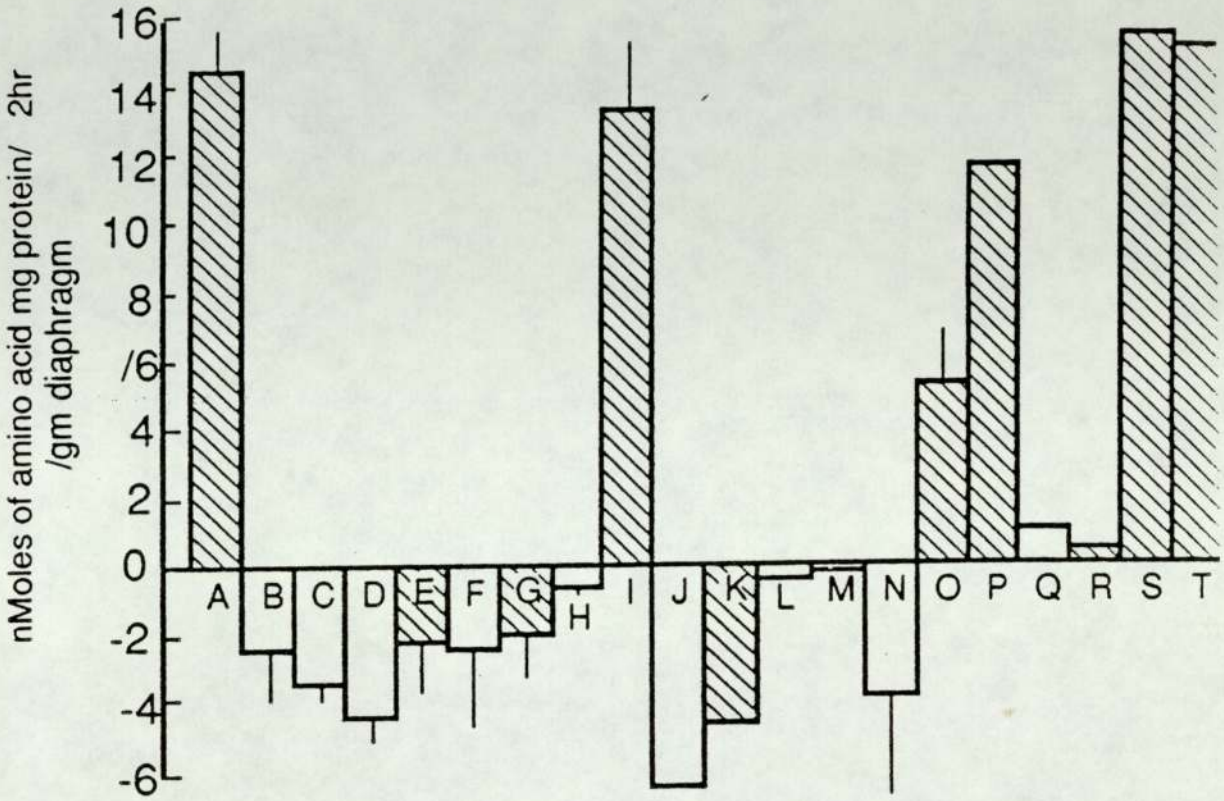
Rate of production of amino acids from mouse diaphragm by the MAC16 tumour homogenate.



Protein content of tumour = 2.9 mg/ml
Weight of diaphragm = 83.0mg
Volume of tumour added per assay = 100µl.

Figure 30

Rate of release of amino acids from mouse diaphragms by tumour homogenates.



A = MAC 16Tumour homogenate
B = MAC 15A cell line
C = MAC 13cell line
D = IOU INSULIN (I)
E = IOUI + MAC 16T
F = 8.0mM 3- HYDROXYBUTYRATE (3-HB)
G = 3 HB(8.0mM) + MAC 16T
H = NaCl 10.4mM
I = NaCl (10.4mM)+ MAC 16T
J = 3-HB(8mM) + IOUI
K = 3-HB(8mM) + IOUI + MAC 16T
L = PhenylmetyhylsulphonylflourideO.5r
M = PMSF(0.5mM) + MAC 16T
N = TRYPSIN INHIBITOR (I) 1.0mg/ml
O = TRYPSIN I + MAC 16T
P = -AMINOCAPROIC ACID 10 mg/ml + MAC 16T
Q = MAC 16T HEATED TO 90°C
R = MAC16T + ACID(perchloric 1.0N)
S = RNA-aSE 1mg/ml + MAC 16T
T = DNA-aSE 1mg/ml + MAC 16T

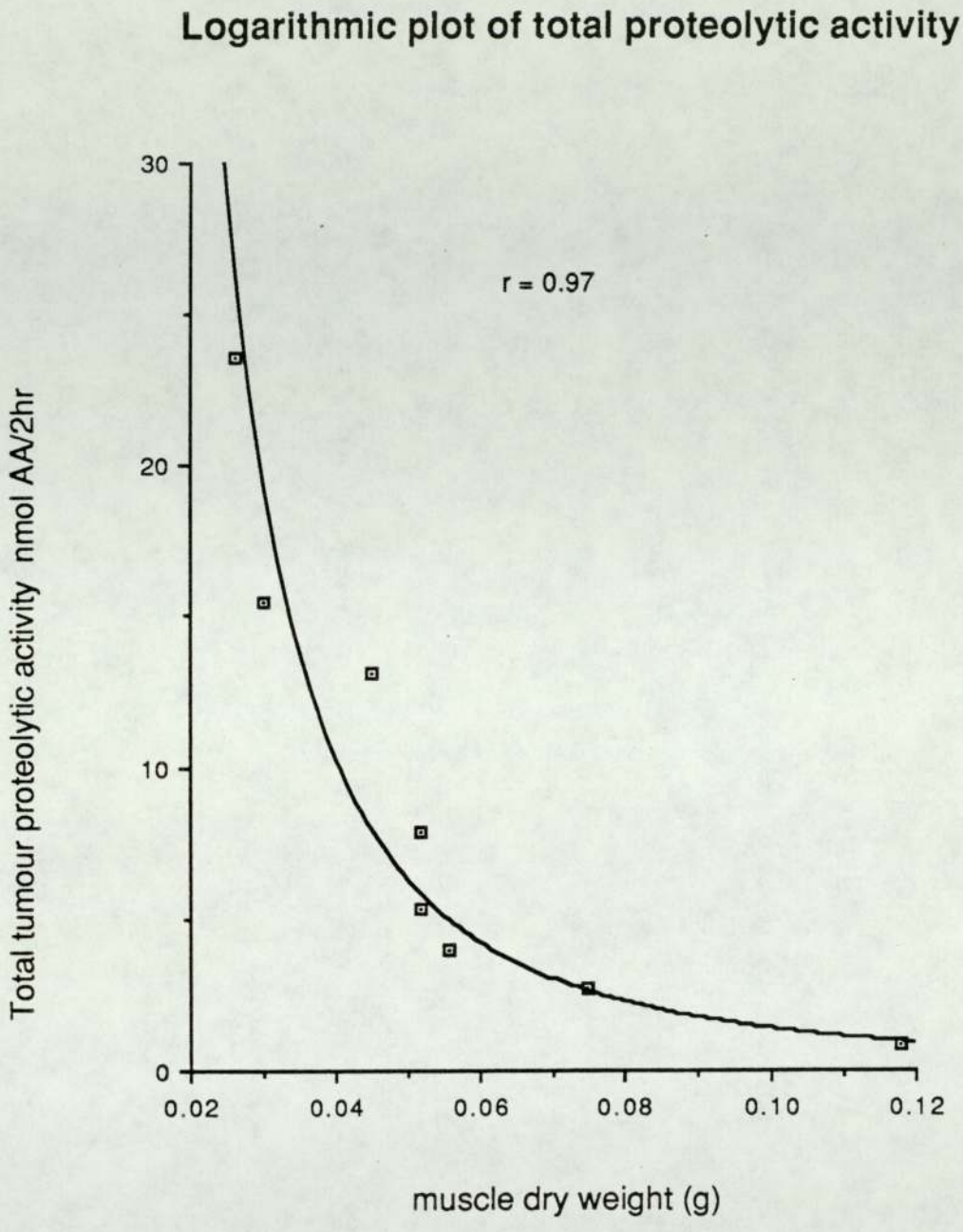
The results are expressed as Mean ± S.E.M of 3 to 13 determinations. *indicates $p<0.05$ from A, **$p<0.001$ from A and +$p<0.001$ from control determined by Students t-test.

E-aminocaproic acid had any effect on the proteolytic actvty produced by the MAC16 tumour. Therefore it is not a nucleic acid nor a plasminogen activator. Small amounts of activity was needed to produce a decrease in muscle dry weight (fig 31). However as total dry muscle weight decreased large amounts of activity were needed to completely deplete the animals muscle stores and as with lipolytic activity there was a logarithmic relationship between the two.

In contrast with the original in vivo MAC16 tumour the cell lines exhibited only lipolytic but no proteolytic activity. This may be due to suppression of proteolytic activity by serum. The lipolytic activity was released into the medium during cell growth (fig 32).

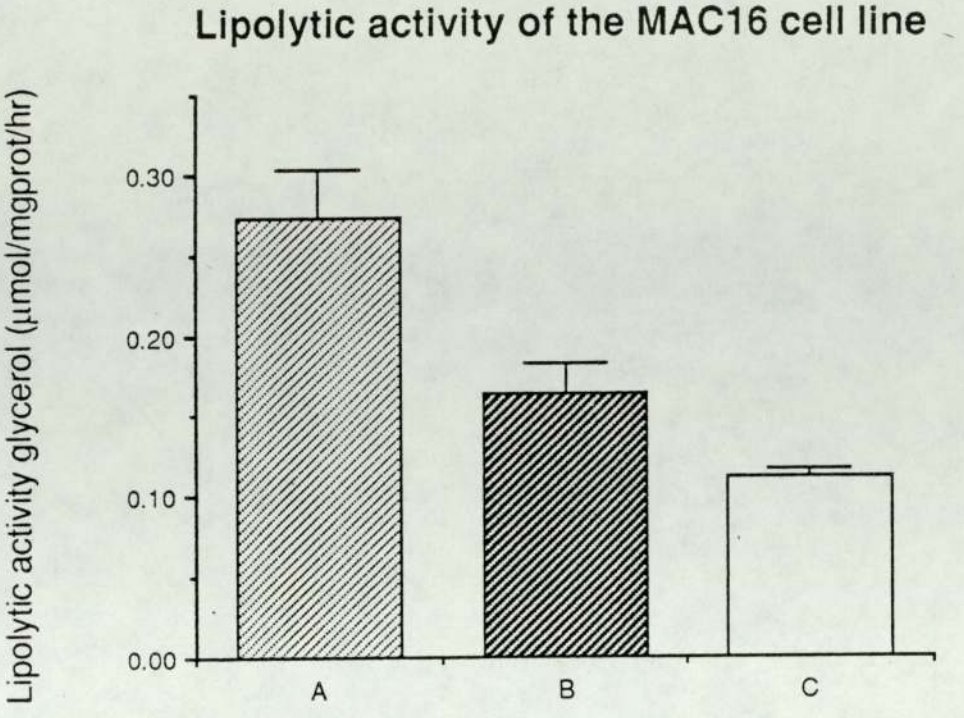
Various prostaglandins exhibit proteolytic activity . Our experiments showed that PGE_2 produces more proteolysis than any of the other prostaglandins studied (fig 33). Tumour necrosis factor is thought to cause proteolysis by its release of PGE_2 (Dayer et al 1985). At high concentrations (10^5U/ml) TNF produced proteolysis in mouse diaphragms and this was inhibited by indomethacin and antitrypsin (fig 34). In contrast indomethacin and antitrypsin only partially suppressed the activity of the proteolytic factor produced by the MAC16 tumour. However when added together they completely abolished the proteolytic activity (fig 34).

Figure 31

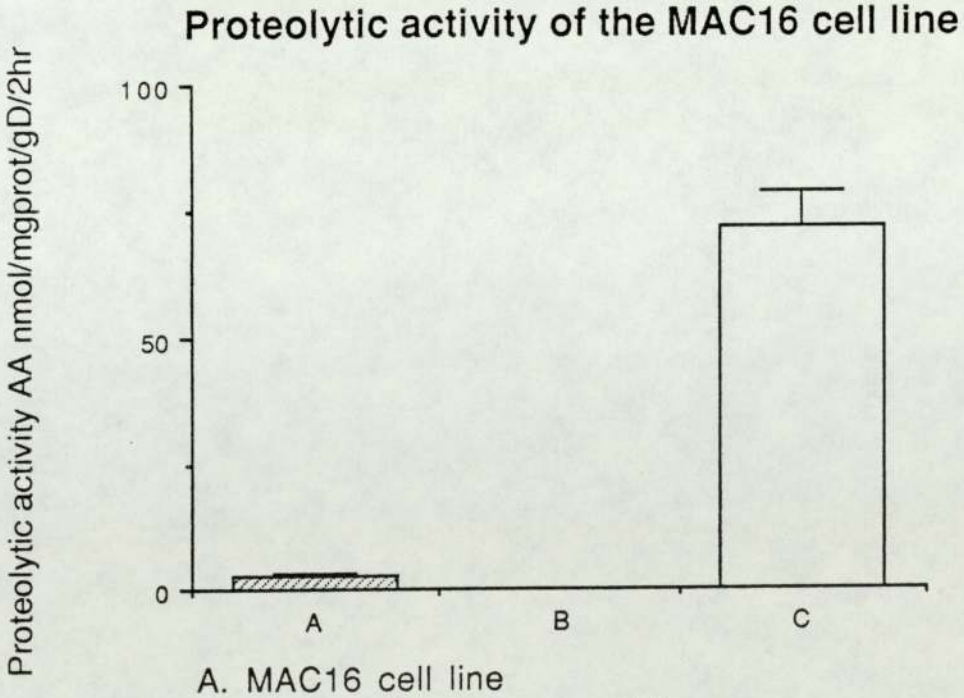


Regression analysis of the relationship between the total tumour content of proteolytic activity and the thigh and gastrocnemius muscle dry weight.

Figure 32



A. MAC16 cell line
B. Media from MAC16 cell line
C. MAC16 tumour in vivo

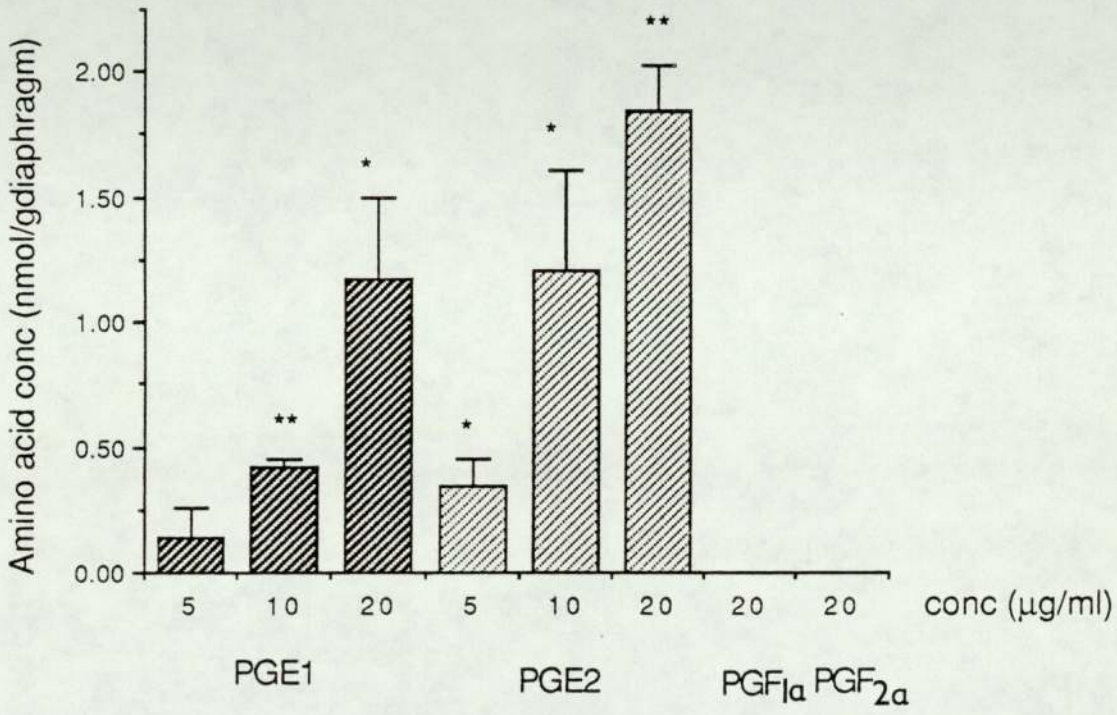


A. MAC16 cell line
B. Media from MAC16 cell line
C. MAC16 tumour in vivo

Results are expressed as the Mean±S.E.M. The number of experiments performed were 3to 13.

Figure 33

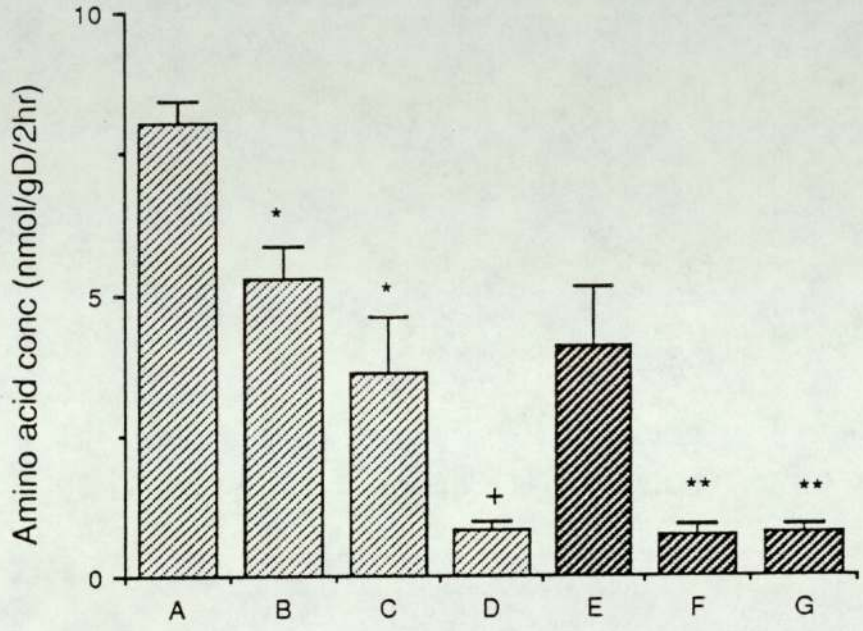
Proteolysis induced in mouse diaphragm by prostaglandin analogues



Results are expressed as the Mean ± S.E.M. The number of experiment performed were 4. Significant difference *p<0.05, ** p<0.005 when compared to control values.

Figure 34

Effect of trypsin inhibitor and indomethacin on proteolysis in mouse diaphragms induced by the MAC16 tumour and byTNF

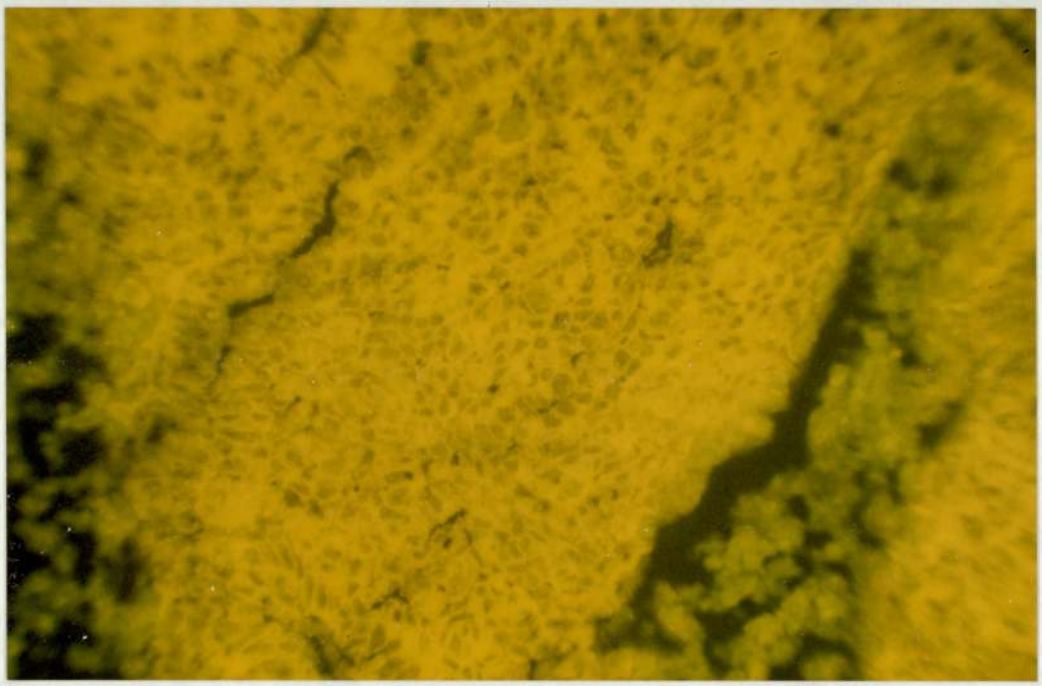


A. MAC16T (0.135mg/assay)
B. MAC16T + 1.0mM indomethacin
C. MAC16T + 1mg/ml Anti-trypsin
D. MAC16T + 1.0mM indomethacin + 1.0mg/ml Antitrypsin
E. TNF 10^5U/ml
F. TNF 10^5U/ml + 1.0mM Indomethacin
G. TNF 10^5U + 1.0mg/ml Antitrypsin

Results are expressed as the Mean ± S.E.M. The number of experiments performed were 3 to 5 . Significant difference * $p<0.05$, + $p<0.005$ when compared to the MAC16 tumour values. ** $p<0.05$ when compared to the TNF value.

In collaboration with Dr F.Steven, University of Manchester the presence of the proteolytic enzyme Guanidinobenzoatase in the MAC16 tumour was examined. This enzyme is a serine protease with characteristics similar to the proteolytic factor found in the MAC16 tumour.

Figure 35



Flourescent micrograph of a MAC16 tumour section stained with 9-aminoacridine and photographed through a yellow interference filter.

Tumour cells posessing guanidinobenzoatase activity appear yellow. The background of connective tissue, intracellular matrix and inactive cells appears green. Thus the MAC16 tumour posseses a large amount of guanidinobenzoatase activity. Proteolytic activity similar to the lipolytic

activity was found in the plasma of MAC16 tumour-bearing animals (table 5). The lack of inhibition of this enzyme by antitrypsin (fig 30) would account for its presence in the plasma. This shows that the MAC16 tumour produces a circulatory proteolytic factor/factors. This enzyme together with the circulatory lipolytic factor/factors may be responsible for the cachectic effects produced by the MAC16 tumour.

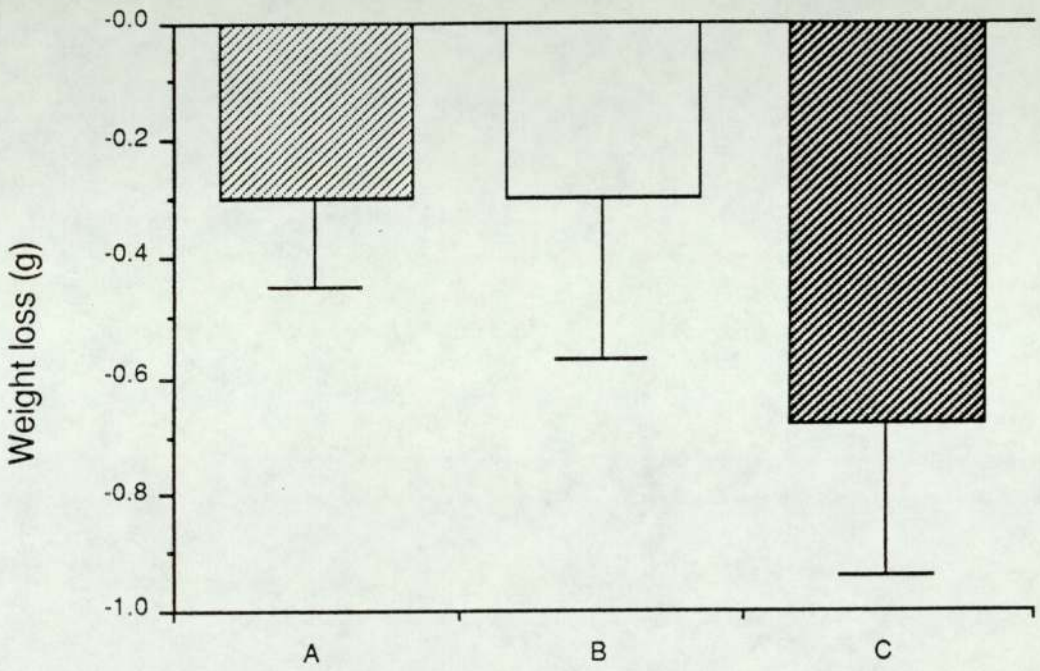
On injection of cell free extracts of the MAC16 adenocarcinoma into non tumour bearing female NMRI mice (initial body weight 20g) weight loss was noted (fig 36). There was no difference in the weight loss produced by an extract of the MAC13 tumour at the same protein concentration from that found with saline injected controls. This suggests that cell free extracts of the MAC16 tumour are capable of producing cachexia. However this was only an experiment with a crude extract and it has been repeated using more purified lipolytic material. The results of this are discussed in the general discussion.

4.2.2.2 Determination of lipolytic activity in other tumour models.

Other experimental animal tumour models were examined for the presence of lipolytic activity. As stated earlier the MAC13 adenocarcinoma is related histologically to the MAC16 adenocarcinoma (Bibby et al 1987) and it displays 10% of the level of lipolytic activity found in MAC16 tumour (table 7). Two human cell lines Raji and GM892 also have low levels of lipolytic activity (table 7). These tumours

Figure 36

Weight loss experienced after IP injections of tumour homogenates



A. control 200µl 0.9% saline.
B. 200µl MAC13 tumour homogenate. (0.280mg/injection)
C. 200µl MAC16 tumour homogenate. (0.265mg/injection)

Results are expressed as the Mean ± S.E.M of 5 animals
There was no significant difference in food and water intake. Weight loss was measured after 6 days.

Table 7

Lipolytic activity of different tumour types

tumour type	strain of mouse	Lipolytic activity µmolglycerol/mgprotei
MAC16 adenocarcinoma	NMRI	0.137±0.0120
MAC13 adenocarcinoma	NMRI	0.013±0.0030
Human hypernephroma	Nude	0.002±0.0004
Raji (Burkitts lymphoma)	cell line	0.011±0.002
GM892 (lymphoblastoid)	cell line	0.013±0.002

have not been reported to cause cachexia in the host. Strain et al (1980) showed a human hypernephroma transplanted into nude mice produced symptoms of cachexia. However in our laboratory the same tumour did not show any of the cachectic symptoms described by Strain. It also had very low levels of lipolytic activity associated with it (table 7).

Human colonic tumours freshly obtained from the operating theatre were also examined. These showed an increased level of lipolytic activity compared to normal colonic tissue from the same patients (table 8). The MAC16 tumour had thirty times the lipolytic activity of normal colonic tissue.

4.2.2.3 Determination of lipolytic and proteolytic activities in the circulating body fluids of mice and humans.

The plasma and urine of MAC16 tumour bearing mice showed high lipolytic activity when compared with non-tumour bearing animals (table 5 and 9). Proteolytic activity was only present in the plasma of tumour bearing animals. The MAC16 tumour had five times as much proteolytic activity as did the MAC13 tumour.

In patient studies no proteolytic activity was detectable in the serum. High lipolytic activity was detected in the serum of cancer patients with weight loss when compared to control subjects (table 10). There was a slight correlation between weight loss and the amount of lipolytic activity in the serum. Alzheimers patients were

Table 8

Lipolytic activities of human colon tumours

Patient	Sex	Age	Weight (kg)	Lipolytic activity µmol glycerol/mgprot
P	female	61	none	1.10±0.20
H	male	86	6.4	0.80±0.20
RE	male	50	12.7	2.30±0.30
A	male	62	none	1.20±0.30
B.C	female	68	none	1.00±0.30
B.C(normal tissue)				0.50±0.10
M	male	68	none	1.00±0.20
M(normal tissue)				0.07±0.04
MAC16 mouse colon adenocarcinoma				0.570±0.500
mouse(normal colon tissue)				0.021±0.004

Table 9

Urine lipolytic activity in tumour bearing and non tumour bearing male NMRI mice.

Tumour type	Lipolytic activity μmol glycerol/10^5 cells /mg creatinine
MAC16 adenocarcinoma	1.060±0.168*
MAC13 adenocarcinoma	0.254±0.014
Non tumour bearing	0.150±0.065

Significant difference *$p<0.001$ compared to non tumour bearing.

Plasma lipolytic activity in untreated, weight losing cancer patients.

Patient	sex	age	tumour type	wt loss (kg)	food intake	blood glucose	lipolytic activity µmol glycerol/10^5cells /ml plasma
J.V.	male	70	prostate	-13.9	decreased	6.4	0.227
R.U.	female	56	ovary	+4.0	decreased	-	0.061
S.K.	male	71	prostate	-4.0	normal	5.4	0.140
G.P.	female	66	ovary /pancreas	-7.7	normal	-	0.087
J.K.	female	60	breast	-12.4	normal	-	0.233
R.D.	male	62	lung	-11.1	normal	4.1	0.370
A.H.	male	64	bladder	-9.0	decreased	-	0.331
A.M.	female	77	lymph nodes	-19.6	norma1	-	0.359

(control values for lipolytic activity for normal subjects range from 0.013 to 0.077 µmoles glycerol/ml plasma)

also studied as they experience vast amounts of weight loss without reduced food intake (Singh et al 1988). These patients had no increase in serum lipolytic activity compared with normal controls. An increase in lipolytic activity was also detected in the urine of cancer patients (table 11). However this had no correlation with the amount of weight loss they had experienced.

4.2.3 Discussion

These results provide evidence for the production of a circulatory lipolytic factor by tumour cells independent of a host response. This factor can be distinguished from those others discussed earlier in the section . The effects seen are unlikely to be due to tumour necrosis factor/cachectin, since there was no evidence of TNF in the MAC16 tumour or the circulation of MAC16 tumour bearing mice either before or after endotoxin administration (Mahony, Beck and Tisdale 1987). The lipolytic factor produced by the MAC16 tumour differs from that reported by Kitada et al (1982) as it does not need to aggregate in the cold before becoming active. In addition the lipolytic activity differs from that described by Masuno et al (1981) which caused a suppression in food and water intake. This factor was also produced by a cell line derived from the MAC16 tumour *in vivo*. The lipolytic factor appeared to be present in three MAC adenocarcinomas irrespective of whether the tumour confers cachexia on its host. However it is the amount of factor present and released that seems to dictate whether or not the animals exhibit cachexia. This factor is probably a

Urine lipolytic activity in untreated, weight losing cancer patients

Patient	sex	age	tumour type	wtloss(kg)	food intake	Lipolytic activity µmolglycerol /10^5cells /ml urine	Lipolytic activity µmolglycerol /10^5cells /mg creatinine
R.D	male	62	lung	9.0	normal	0.540±0.058	2.18±0.23
J.C	female	63	cervical	0	increased	0.563±0.058	6.95±0.72
M.T	female	62	ovarian	18.3	decreased	0.500	-
E.H	male	82	prostrate	17.5	normal	0.424±0.072	5.36±1.36
B.T	female	53	ovarian	31.0	decreased	0.510±0.026	4.43±0.23

(control values for lipolytic activity for normal subjects range from 0.07to 0.240µmoles glycerol/ml urine or 0.5 to 1.88 µmoles glycerol/mg creatinine).

protein as it is nondialysable, and destroyed by heat and acid. It is not a nucleic acid as it is not degraded by RNAase or DNAase. It is not a prostaglandin as its production was not inhibited by indomethacin. The lipolytic activity was not due to a nonspecific proteolytic effect as neither trypsin inhibitor or phenylmethylsulphonylfluoride caused a significant reduction in lipolysis. Insulin a potent antilipolytic hormone reduced the lipolytic activity of the MAC16 tumour. Ketone bodies have also been shown to directly reduce lipolysis in adipose tissue (Bjorntorp, 1966). Thus in vitro 3-hydroxybutyrate inhibited the lipolytic activity of the MAC16 tumour and this effect was additive with insulin. Therefore the lipolysis seen with the MAC16 tumour responds to normal physiological stimuli. Medium chain triglycerides (MCT), capable of elevating plasma ketone body levels have been reported to prevent weight loss induced by the MAC16 tumour and to reduce tumour size (Tisdale and Brennan 1988). These effects may be due to inhibition of the lipolytic factor produced by the MAC16 tumour. The factor appears to act differently from adrenaline produced in fasting, since propranalol had no effect on the lipolytic activity. The presence of this lipolytic factor in the serum and urine indicates it produces a circulatory effect rather than a local effect. It may be produced to mobilise the hosts lipids required for the tumour growth.

In addition, there is evidence for the production of a proteolytic factor/factors by the MAC16 adenocarcinoma. This proteolytic activity was unique to the MAC16 tumour and

was not found in measurable quantities in other MAC tumours unlike the lipolytic activity. The proteolytic activity was also inhibited by both insulin and 3-hydroxybutyrate. Insulin has been shown in a similar system to stimulate protein synthesis and inhibit protein degradation (Fulks et al 1975). Ketone bodies have been shown to cause a decrease in proteolysis in isolated rat diaphragms (Palaioglogos and Felig 1976). As insulin and 3-hydroxybutyrate have inhibited both factors it may explain their ability to reverse experimental cachexia (Moley et al 1985, Tisdale, Brennan and Fearon 1987). This is discussed in more detail in section 4.7. The proteolytic factor appears to be a serine protease as activity was completely abolished by phenylmethylsulphonylfluoride which reacts with serine hydroxyl groups. It is not a plasminogen activator or nucleic acid as E-aminocaproic acid, RNAase and DNAase had no effect on the proteolysis. A partial reduction in response was seen with both a trypsin inhibitor or indomethacin. Hence the proteolytic activity seen may be due to a number of actions including the production of a trypsin-like serine protease and the stimulation of prostaglandin E_2 production. Lambombardi et al (1983) have reported a trypsin-like serine protease produced by the Walker 256 carcinoma cells. Another trypsin-like protease identified was guanidinobenzoatase which we have shown to be present in the MAC16 adenocarcinoma. Whether this enzyme was responsible for the proteolysis seen remains to be determined. The proteolytic factor was also present in the circulation so producing a circulatory effect rather than a

local effect. Therefore the cachectic effect of the MAC16 tumour appears to be due to the production by the tumour of catabolic factors.

Further experiments showed that lipolytic activity was associated with various different murine and human tumour types. Studies on the serum and urine of cachectic cancer patients has shown the presence of high lipolytic activity when compared with control subjects.

The production of lipolytic factors in control subjects after a period of starvation is discussed in the next section.

4.3 The production of lipolytic factors in starvation.

4.3.1 Introduction

In starvation (as described earlier in the general introduction) glycogen stores are depleted and survival depends largely on energy derived from fat stores. As a consequence of the increased fatty acids available to the liver ketone bodies (3-hydroxybutyrate, acetoacetate and acetone) are produced.These ketone bodies can be utilised as an energy fuel by the brain when the concentration becomes high enough thus reducing the requirement for glucose. Another route followed in starvation to supply fuel to the brain is that of muscle degradation to produce amino acids which happens only in the early stages of starvation. These amino acids are then converted by the liver to glucose. In fasting the blood glucose and insulin levels fall to approximately 80mg/dl and 10units/ml respectively. This triggers hormones to be released such as adrenaline and glucagon. These are lipolytic hormones, that stimulate hormone sensitive lipase to break down triglycerides into free fatty acids which are further converted into ketone bodies in the liver.

In addition to hormonal action in starvation it has been reported that lipolytic factors are produced to mobilise fat depots. As early as 1947 Weil and Stetten identified a fat mobilising substance in the urine of fasting rabbits. This substance was not present in rabbits fed on a normal diet. They suggested a possible relationship of this substance with the pituitary peptide

adipokinin. In addition Chalmers et al (1958) studied urine collected from healthy human subjects after a 36hr period of fasting. The urine extract was then injected into mice and the effect on lipid mobilisation studied. They concluded that there was a lipid mobilising substance present in fasted subjects. This substance was not present in the same subjects when they were fed a normal diet. Two patients with neoplastic disease were also studied and found to have a fat mobilising substance in their urine. It was not determined whether this substance was the same fat mobilising substance as found in normal healthy subjects. They further characterised this substance showing it to be a polypeptide of pituitary origin resembling corticotrophin (Chalmers et al 1960). Liebelt et al (1971)have postulated the production of another lipid mobilising factor by the CBA 2663 stomach tumour grown in GTG-obese mice. Evidence has been presented for the existence of this factor in the urine of starved animals and humans. Isolation of this substance has not been carried out.

Identification of similar lipolytic factors in the serum of fasting control subjects has not yet been reported.

4.3.2 Results

We have studied the effect of starvation in mice and control human subjects after fasting over a 24 h period. A small but significant reduction in blood glucose level and in plasma free fatty acid levels were seen in the fasted mice (table 12). Body composition analysis showed a slight increase in dry muscle weight compared to MAC16 tumour

bearing animals. However there was still a significant reduction in carcass fat (table 12). Body fluids of the mice were examined for lipolytic activity. It was noted that there was no increase in lipolytic activity in the urine compared to control fed mice. There was a slight increase in plasma lipolytic activity but still almost ten times less than seen in the MAC16 tumour bearing mouse (table 13).

In the human control subjects again no increase was seen in urine lipolytic activity after a 24h starvation period. However, similar to the mice, an increase in plasma lipolytic activity was noted (table 13). This was not as great as seen in any of the cachectic cancer patients. The lipolytic factors present in the urine and plasma after starvation were examined in more detail to determine if they were the same factors present in the MAC16 adenocarcinoma (discussed later).

Table 12

Effect of 24hr starvation on male NMRI mice.

	Weight loss (g)	Blood glucose mg/dl	Plasma FFA mg/dl	Dry wt muscle(g) (thigh + gastrocnemius)	Fat(g)
control NMRI	0.00±0.00	136.00±5.00	29.0±2.0	0.090±0.003	1.70±0.09
NMRI after starvation	6.16±0.21**	106.04±10.12*	10.0±1.0**	0.077±0.003*	0.54±0.15**
MAC16 tumour bearing (21 days after transplantation)	5.40±0.60**	108.00±11.00*	10.0±1.0**	0.070±0.002*	0.58±0.11**

The results are expressed as the Mean±S.E.M. The number of animals used were 6 to 8. Significant difference *$p<0.05$, **$p<0.005$ compared to control NMRI mice. Initial weight of the mice were approximately 26 to 28g.

Table 13

To determine the production of lipolytic factors during starvation

(a) 24hr starvation of male NMRI mice

	urine lipolytic activity mM glycerol /mg creatinine	plasma lipolytic activity mM glycerol /ml plasma
control NMRI	0.150±0.065	0.020±0.003
24hr starvation	0.140±0.065	0.035±0.005
MAC16 tumour	1.060±0.168	0.201±0.030

(b) 24hr starvation of human control subject(A.S)

	urine lipolytic activity mM glycerol /mg creatinine	plasma lipolytic activity mM glycerol /ml plasma
normal diet	0.056±0.004	0.07±0.02
24hr starvation	0.048±0.008	0.150±0.03
cachectic cancer patient(B.T)	0.218±0.023	0.370±0.030

The results are expressed as the Mean±S.E.M. The number of animals studied were 6 to 8.

4.3.3 Discussion

Mice, after a 24h starvation, show a decrease in blood glucose and a dramatic reduction in carcass fat. This is due to the body fat stores being broken down to supply the fuel needed for the brain. There was a slight decrease in muscle dry weight, probably due to some degradation to provide amino acids. These amino acids are then converted in the liver to glucose the major fuel source used by the brain.

Contrary to results from other laboratories we found no increase in lipolytic activity in the urine of animals and man after fasting. This may be due to the time period that starvation was studied over. We only studied starvation over a 24h period while most other reports used a period of at least 36h of fasting. The increase in lipolytic activity in the plasma of animals and man was probably due to an increase of normal lipolytic hormones stimulated by fasting. This increase was much less than the values seen in cachectic cancer patients described in section 4.2. Determination of whether the lipolytic activity seen in starvation and/or cachectic cancer patients was due to the same factors that are present in the MAC16 adenocarcinoma is discussed in section 4.5. Further characterisation and purification of the factors produced by the MAC16 adenocarcinoma is discussed in the next section.

4.4 Purification of the lipolytic and proteolytic factors produced by the MAC16 adenocarcinoma.

4.4.1 Introduction

All separation procedures involve the distribution of components in a mixture between two phases which subsequently can be separated mechanically. There are various properties that can be used to allow separation to take place. A simple method of separation is by precipitation. This is dependent on the solubility difference between the components.

Size is an important property in that it allows substances to be separated by their molecular weight. Using gel filtration chromatography the molecular weight of particular molecules can be determined and these molecules identified.

Another characteristic a molecule has that can be used in the separation process is the charge it carries. Using techniques such as ion exchange and isoelectric focussing molecules can be separated due to their charge. The basic theory underlying all these techniques is that unlike charges attract and like charges repel, thus the more charged a species is the further it will be attracted to an unlike source.

In many column chromatographic procedures hydrophobicity is the property that separates the substances being investigated. Columns consist of varying lengths of carbon chain from C18 to C2 with decreasing hydrophillicity. Thus a compound will be retained for

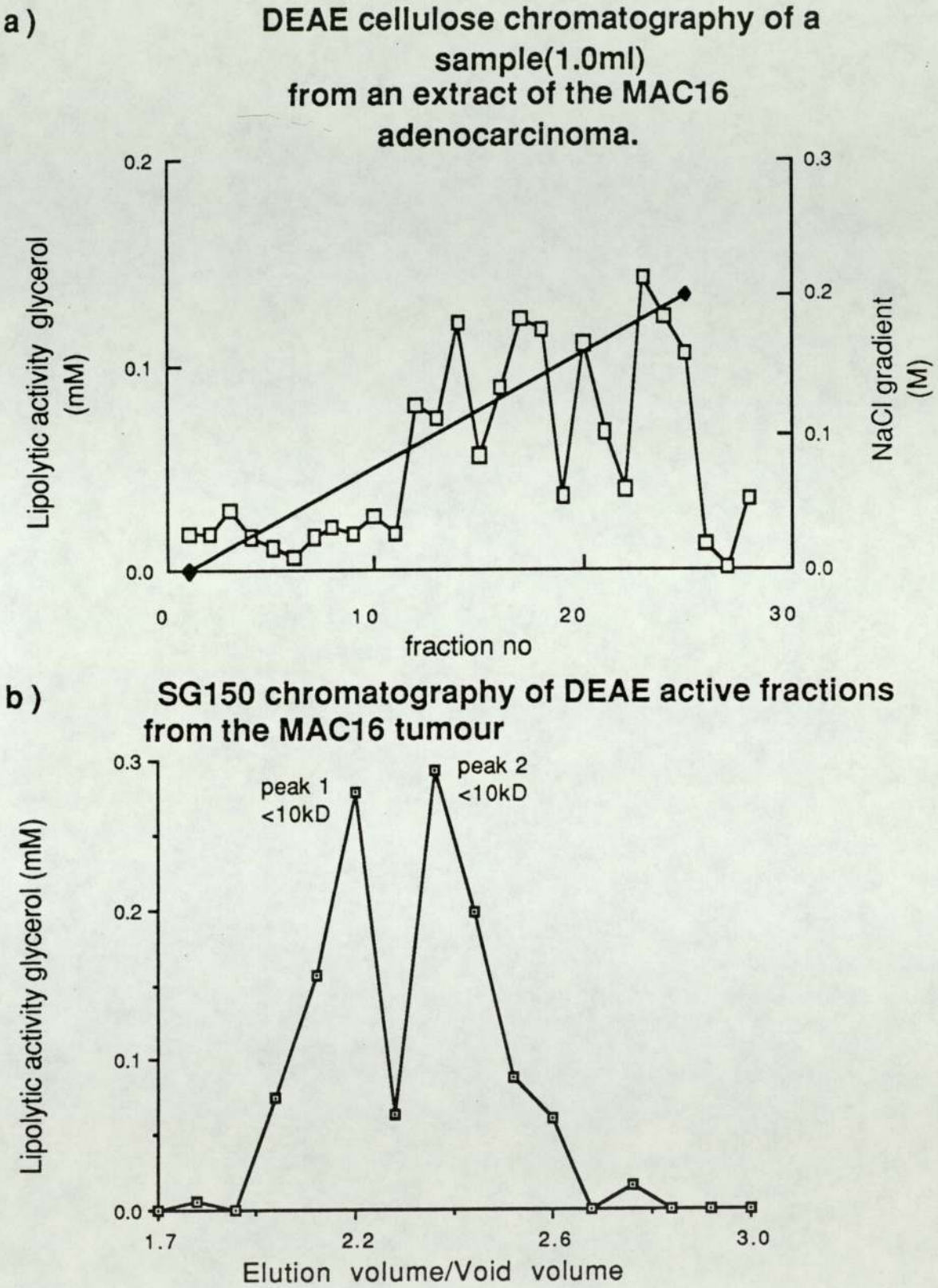
longer the more hydrophobic its nature is.

All of these separation procedures have been used at some time in the isolation and purification of the catabolic factors produced by the MAC16 adenocarcinoma.

4.4.2 Results

Lipolytic activity in low speed supernatant fractions of the MAC16 tumour was retained on a DEAE cellulose and was eluted as four peaks of lipolytic activity under the influence of a salt gradient (0 to 0.2M NaCl) (fig 37). These peaks eluted at salt concentration of 0.075M, 0.09M, 0.105M and 0.13M i.e. between 0.1-0.2M of the NaCl gradient. The cytosolic extract also gave four peaks of proteolytic activity retained by a similar anion exchange column and eluted by a NaCl gradient (0 to 0.2M) (fig 38). These peaks eluted at 0.095M, 0.12M, 0.14M and 0.15M NaCl i.e between 0.08-0.15M of the NaCl gradient. Hence there was an overlap between the lipolytic and proteolytic activities of the MAC16 tumour. After DEAE cellulose to seperate these activities Sephadex G150 molecular exclusion chromatography was used. The lipolytic activity of the MAC16 tumour eluted as 2 peaks of activity at an elution volume/void volume between 2.0-2.6 (fig 37).Both of these peaks were of apparent molecular weight < 10k daltons. The same profile was seen with each DEAE peak. The proteolytic activity also eluted in 2 peaks. A large peak of activity at an elution volume/void volume between 0.87-1.30 (fig 40) and a much smaller peak at an elution volume/void volume between 2.5-2.7. Therefore the molecular weight of the proteolytic

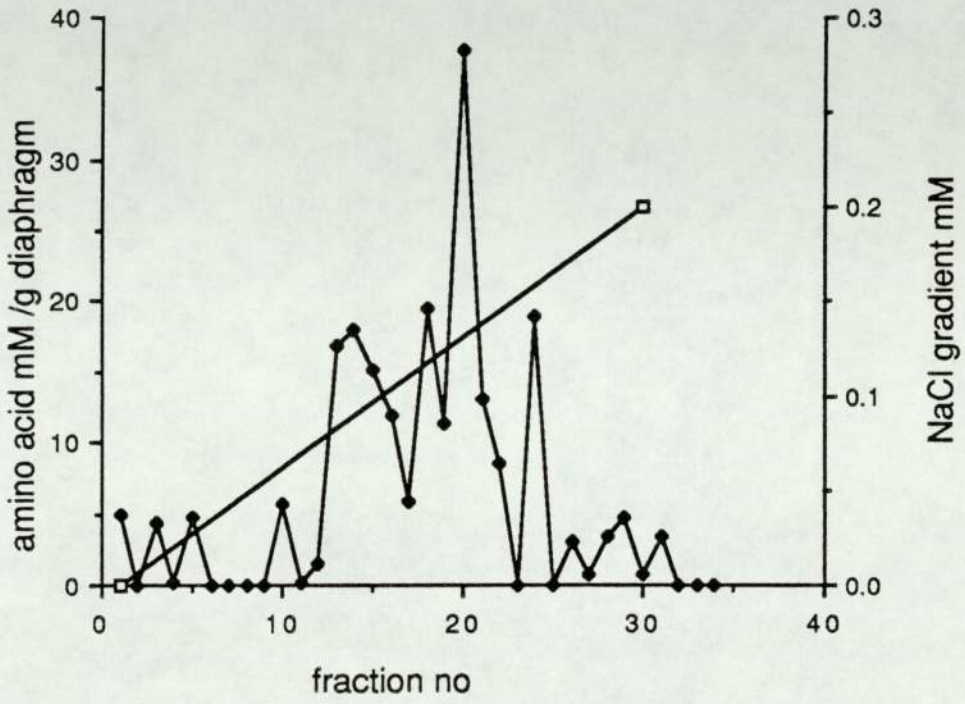
Figure 37



Lipolytic activity distribution patterns of 1.0ml fractions obtained from a) DEAE column and b) Sephadex column (void volume 23.0ml). Protein content of tumour = 2.8mg/ml.

Figure 38

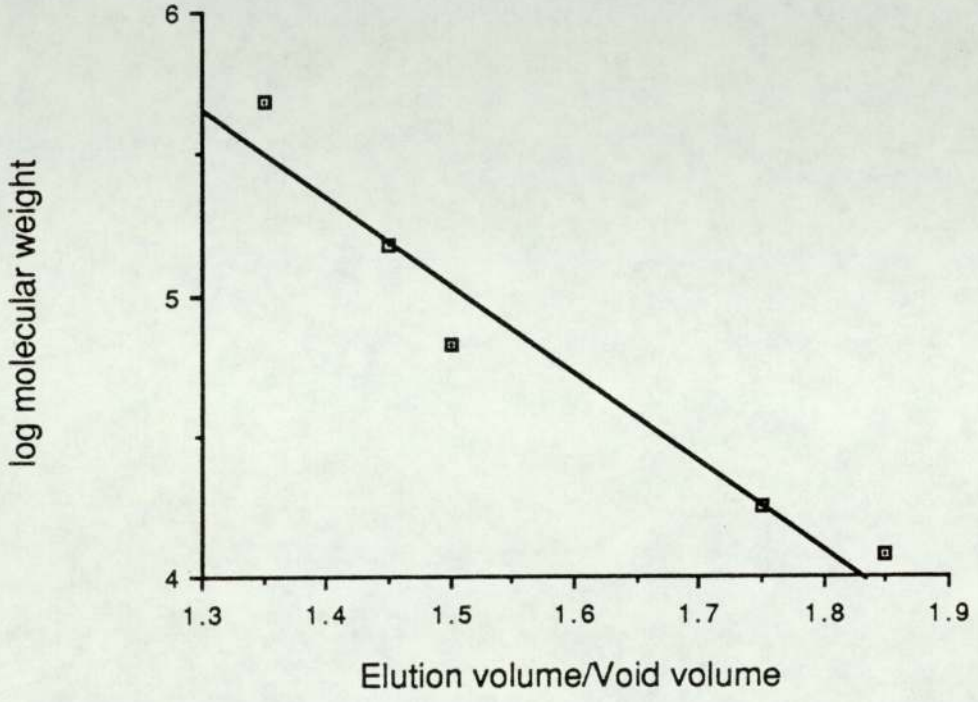
DEAE cellulose chromatography of a sample (1.0ml) from the MAC16 tumour homogenate



Proteolytic activity distribution pattern of 1.0ml fractions obtained from a DEAE column in a phosphate buffer pH8.0 followed by a 0-0.2M NaCl gradient. Protein content of the tumour = 2.8mg/ml.

Figure 39

Calibration graph of the Sephadex G150 column

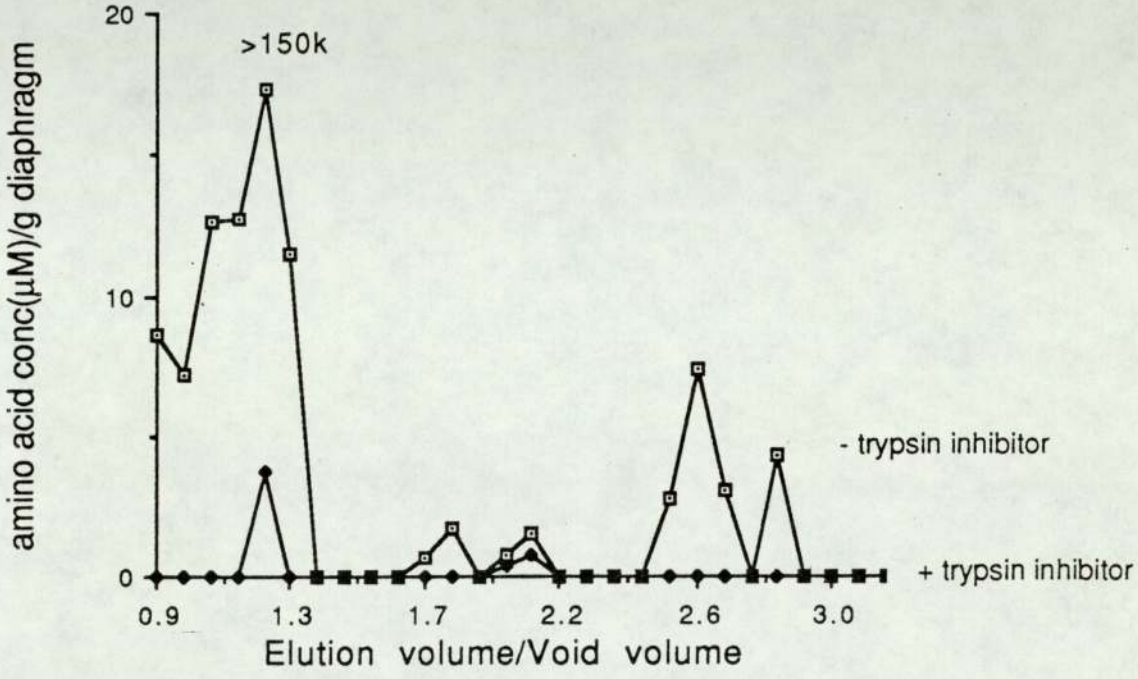


Molecular weight markers used:

Cytochrome C 12000 daltons
Myoglobin 17800 daltons
Albumin 66000 daltons
Alcohol dehydrogenase 150000 daltons
Apoferritin 480000 daltons

Figure 40

SG150 chromatography of DEAE active proteolytic fractions with and without trypsin inhibitor.



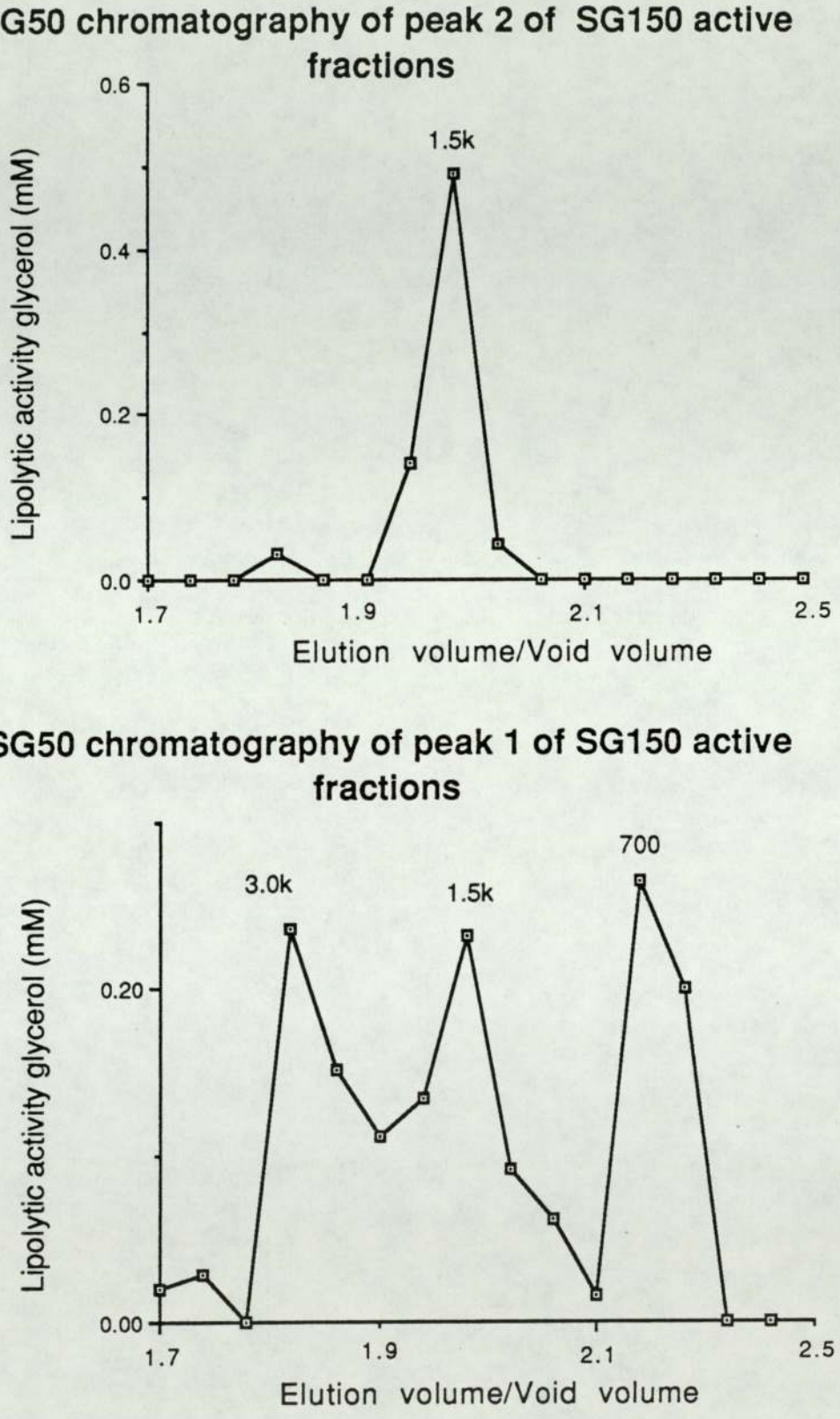
Proteolytic activity distribution pattern of 1.0ml fractions obtained from G150 sephadex column eluted with 0.01M phosphate buffer ,pH8.0.

activity (>150k daltons) is much greater than the lipolytic activity. The proteolytic activity was destroyed by the addition of a trypsin inhibitor (fig 40). To achieve better resolution of the lipolytic factors sephadex G50 chromatography was used. Peak 2 from the SG150 resolved into a single peak of apparent molecular weight of approximately 1500 daltons. Peak 1 from the SG150 resolved into 3 peaks of apparent molecular weight of approximately 3000, 1500 and 700 daltons respectively (fig 41).

Crude supernatant fractions from the MAC13 adenocarcinoma was also chromatographed in the same way but using a more concentrated sample. The elution profile on DEAE cellulose was similar to that produced with the MAC16 homogenate with four peaks of lipolytic activity eluted at salt concentrations 0.08M, 0.1M, 0.13M and 0.16M (fig 43). Sephadex G50 chromatography was carried out on the MAC13 tumour homogenate. This showed two of the same peaks of lipolytic activity found in the MAC16 tumour with apparent molecular weights 1500 and 700 respectively (fig 43).

Following this initial stage reverse phase high pressure liquid chromatography was used for further purification. Many problems were found with this especially with finding a suitable material that will retain the lipolytic factors. Numerous supports were tried but with all at least some of the activity eluted in the solvent phase at fraction 0/1 (table 14). Hence the factors are polar substances and highly hydrophillic. The best seperation was achieved with a C4 butyl column (fig 44). Unfortunately there were no peaks of absorption on the hplc

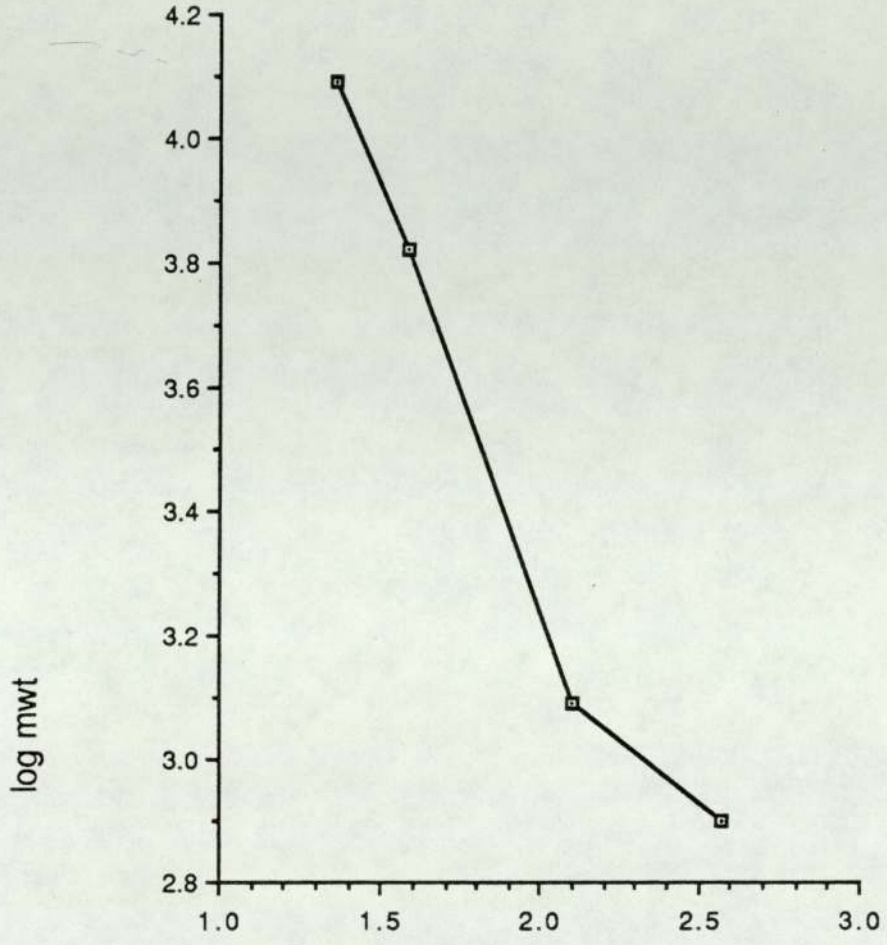
Figure 41



Lipolytic activity distribution pattern of 1.0ml fractions obtained from a G150 sephadex column run in phosphate buffer pH8.0. Void volume = 17.0ml.

Figure 42

Calibration graph of the Sephadex G50 column



Molecular weight markers used:

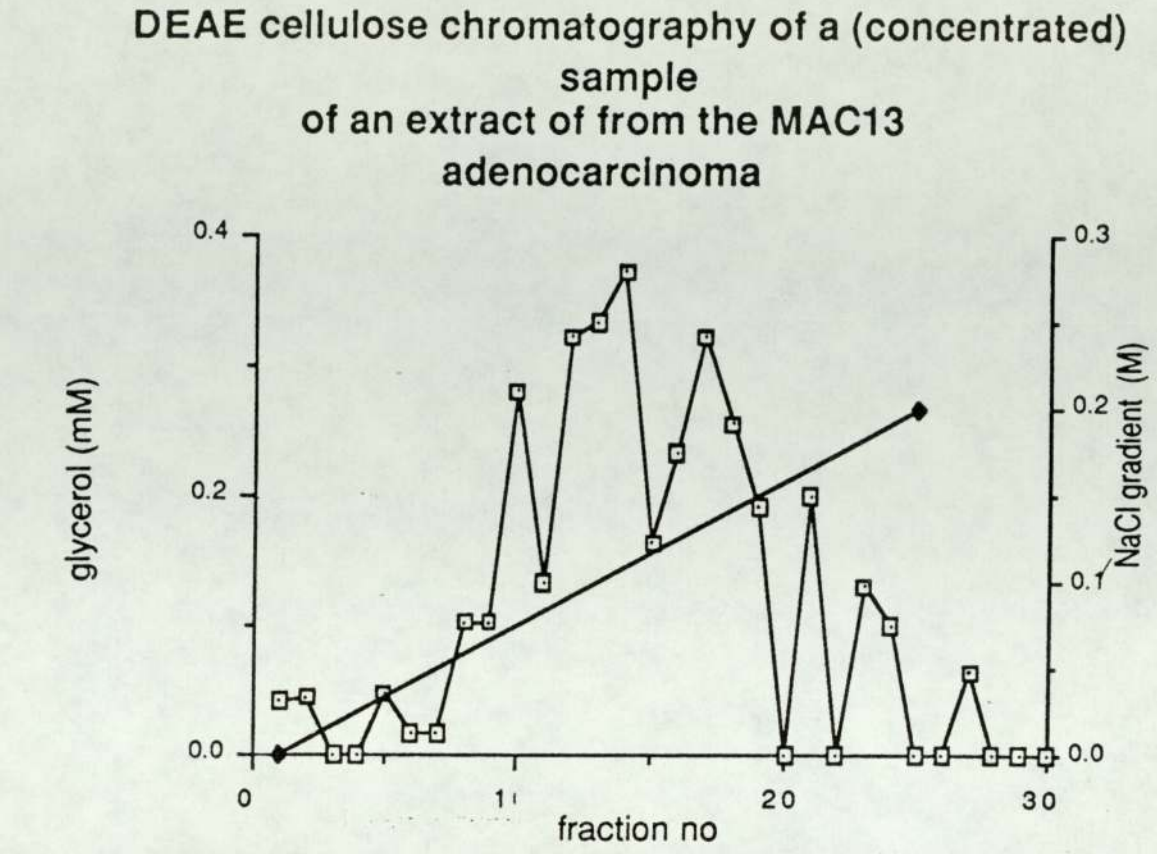
Rifampicin 834 daltons

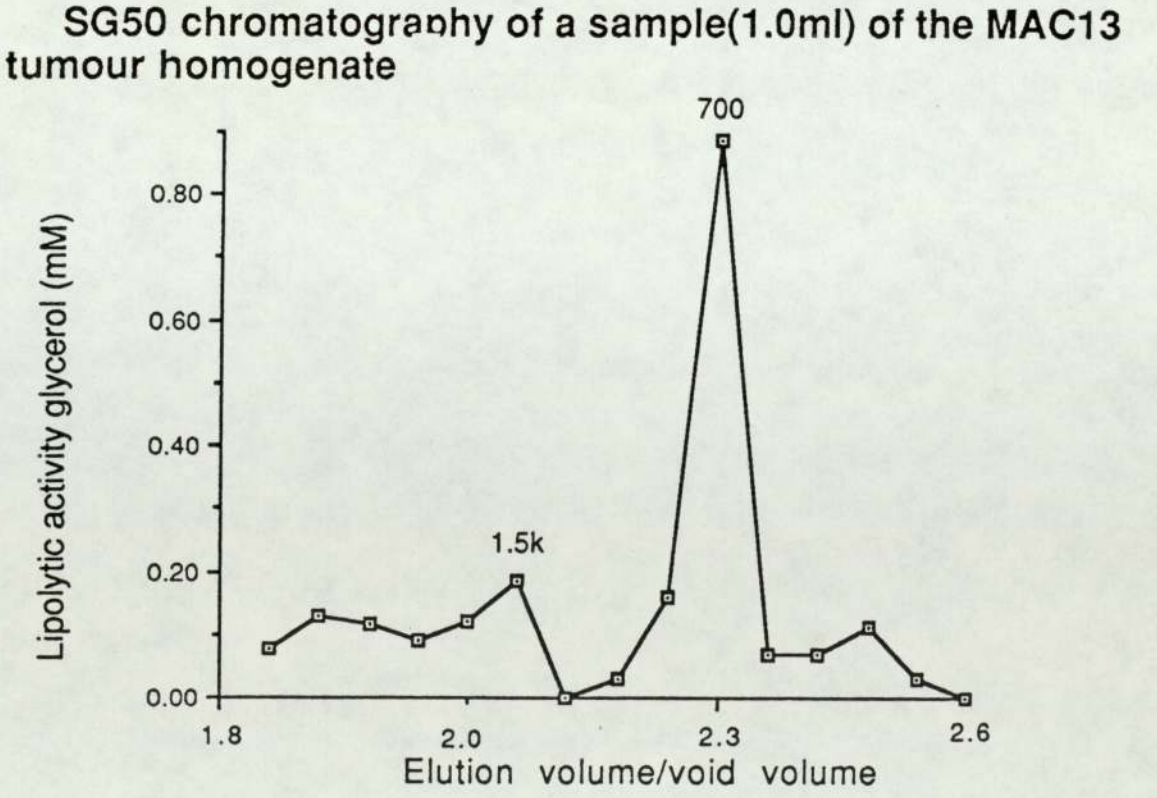
Actinomycin D 1247 daltuns

Aprotinin 6600 daltons

Cytochrome C 12400 daltons

Figure 43





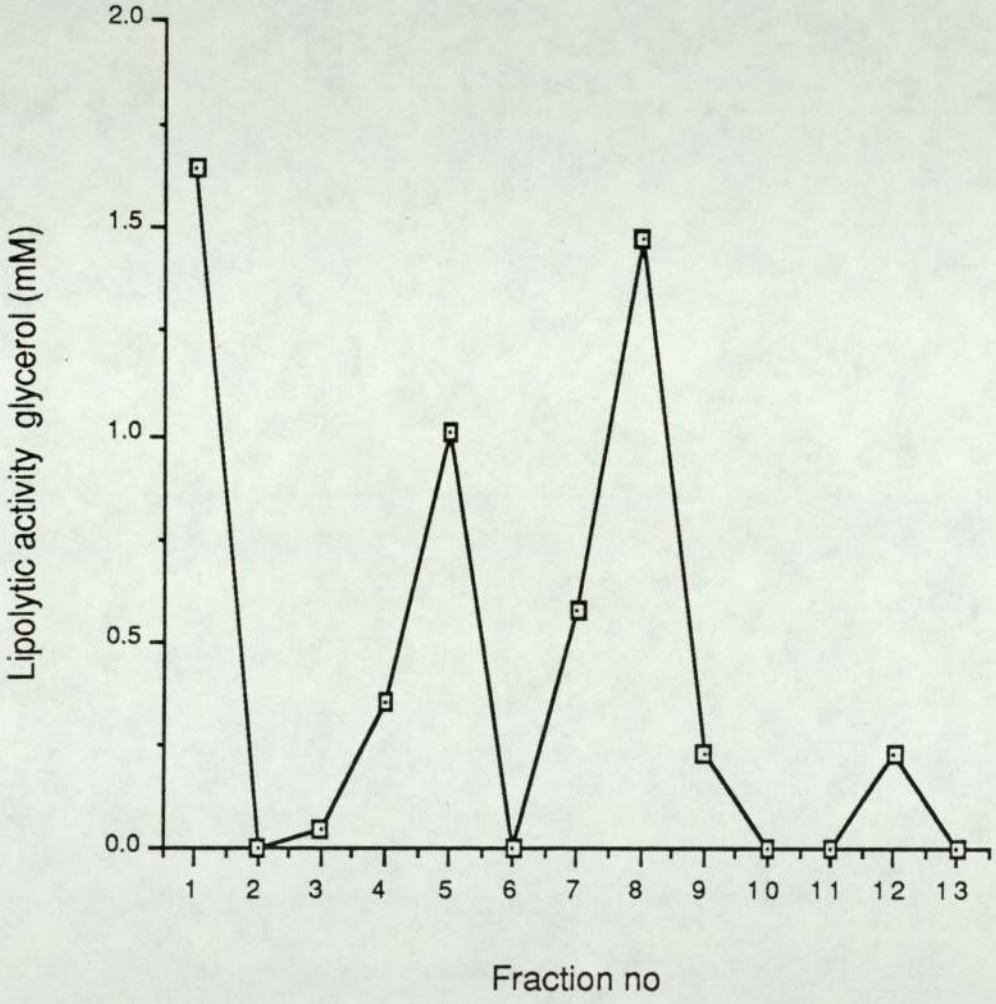
Lipolytic activity distribution patterns of 1.0ml fractions obtained from a) DEAE column and b) Sephadex column void volume=17.0ml. Protein content applied to column = 4.0mg.

Table 14

Column used	Conditions	Factor eluted at 0/1 fraction no
C18	10-60%ACN	* *
C8	10-60%ACN/TFA	** +fraction13
CN	10-60%ACN/TFA	* *
CN	0-15%ACN/TFA	* *
AMINO	10-65%ACN/H_2O	**+some eluted with sodium acetate
CON A sepharose	0.028M Na phosphate	* *

Figure 44 (a)

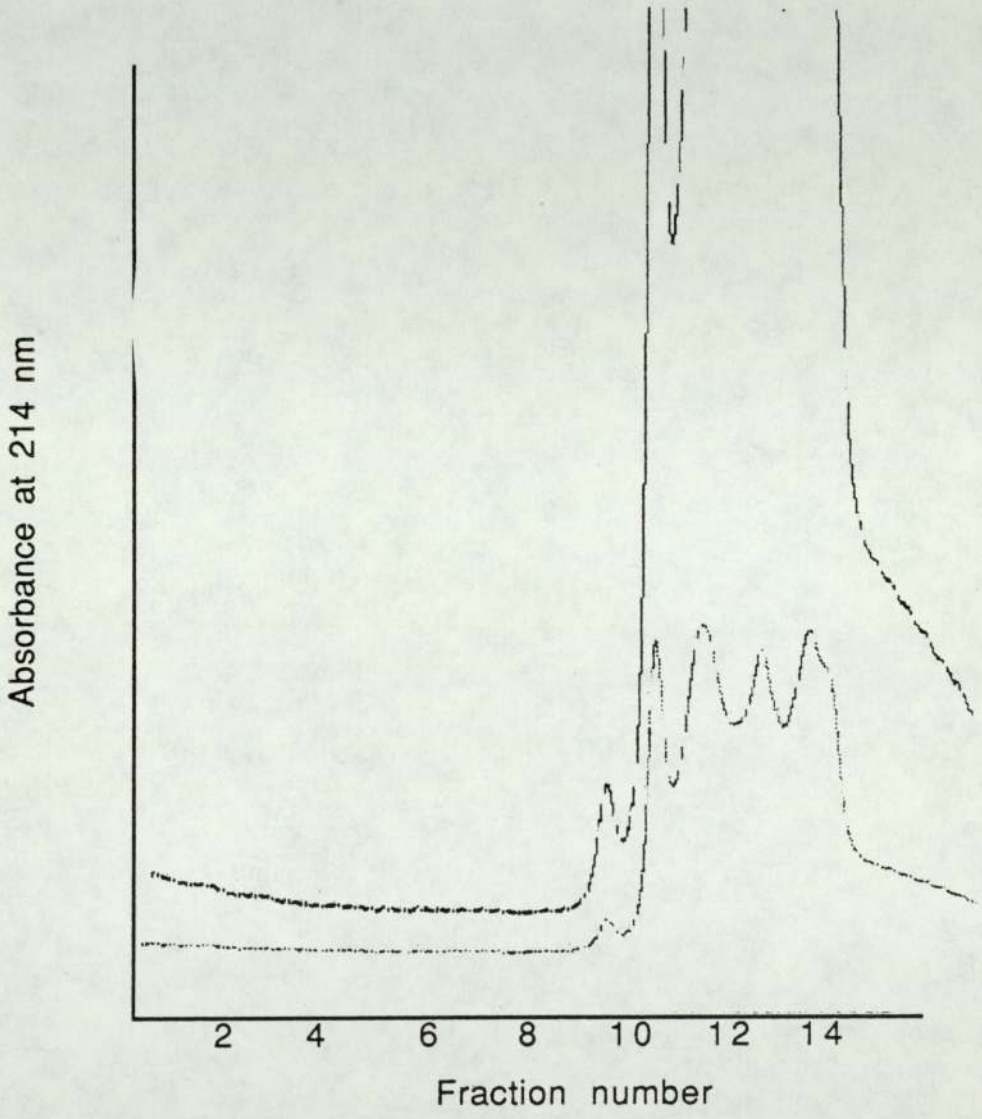
H.P.L.C: C4 hydrophobic chromatography of partially purified lipolytic factor



Lipolytic activity distribution pattern of 0.4ml fractions obtained from a C4 column run on a 30min gradient of 10 to 60% acetonitrile : H20/ TFA(0.1%). Run at a rate of 0.2ml/min. The three peaks correspond (respectively in order of elution) to molecular weights of 3, 1.5, and 0.8kD as measured by Sephadex G50 chromatography.

Figure 44 (b)

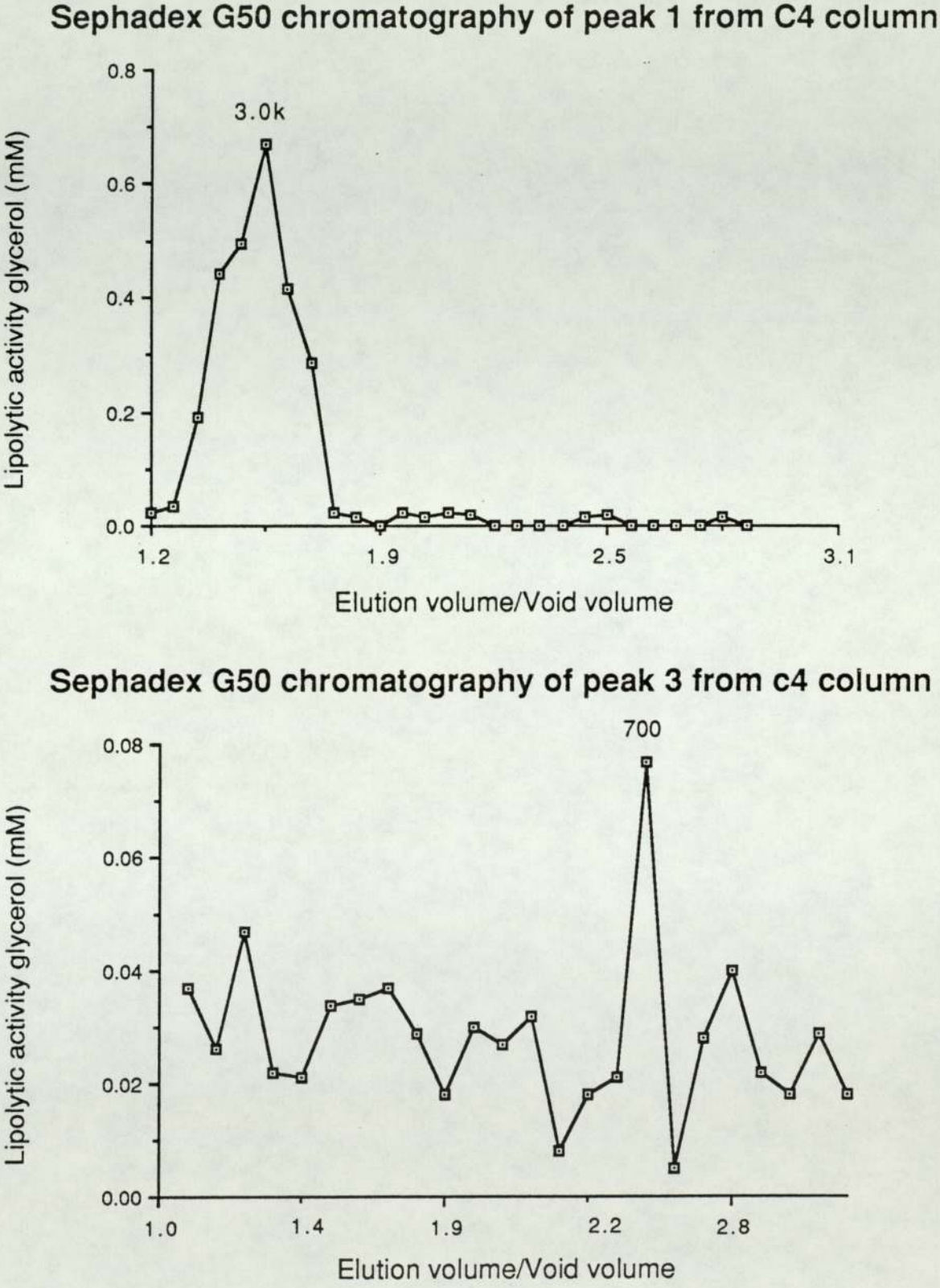
Hplc trace of C4 hydrophobic chromatography.



trace that related to the active peaks after bioassay (trace 44). The active peaks from the C4 column were subjected to Sephadex chromatography to determine the molecular weights of each peak (fig 45). Peak one was of approximate molecular weight 3.0k daltons, this was then passsed down a C4 column again and peaks of all three molecular weights were obtained (fig 46). This indicates a relationship between all three species of lipolytic factor. A P4 biogel column was used for desalting purposes but the resolution of activity was not as good as Sephadex G50 (fig 47) and again nothing was seen on the hplc trace (trace 47). Ultimately a DE anion exchange column was used, the resolution of peaks of activity was good (fig 48). Unfortunately again no peaks of absorption were found on the hplc trace (48) that related with active peaks after bioassay. Thus the amount of factor present in our sample is very small. For further analysis greater amounts of the factors are needed, and collection of more of the material is now in progress. An absorbance scan of the crude factor showed peaks at 260 and 211nm (fig 49).

Further experiments were caried out on the partially purified lipolytic material. This material was not destroyed by heat (90°C for 15min), acid (sulphuric acid 1.0N), RNAase(1.0mg/ml) nor DNAase (1.0mg/ml). It was not degraded by periodate treatment. The effect of acid on the partially purified factor produced by the MAC16 tumour depends on the type of acid used. Perchloric acid (1.0N) inhibited the effect of the factor, however, sulphuric acid (1.0N) did not effect the factors lipolytic activity. With perchloric acid the sample was neutralised with KOH (20%W/V)

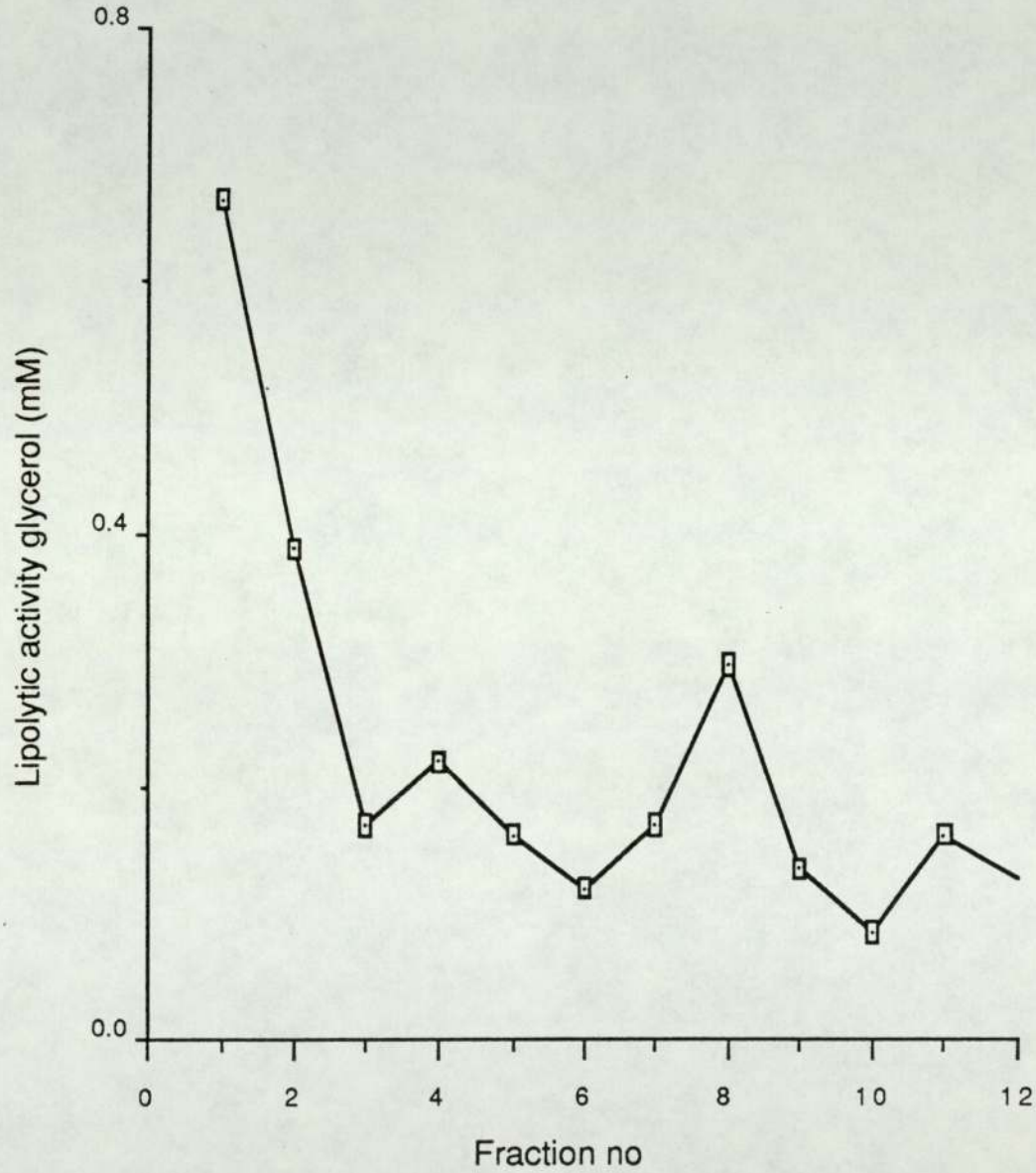
Figure 45



Lipolytic activity distribution pattern of 1.0ml fractions obtained from a G50 Sephadex column eluted with phosphate buffer pH 8.0. Void volume = 16.0ml.

Figure 46

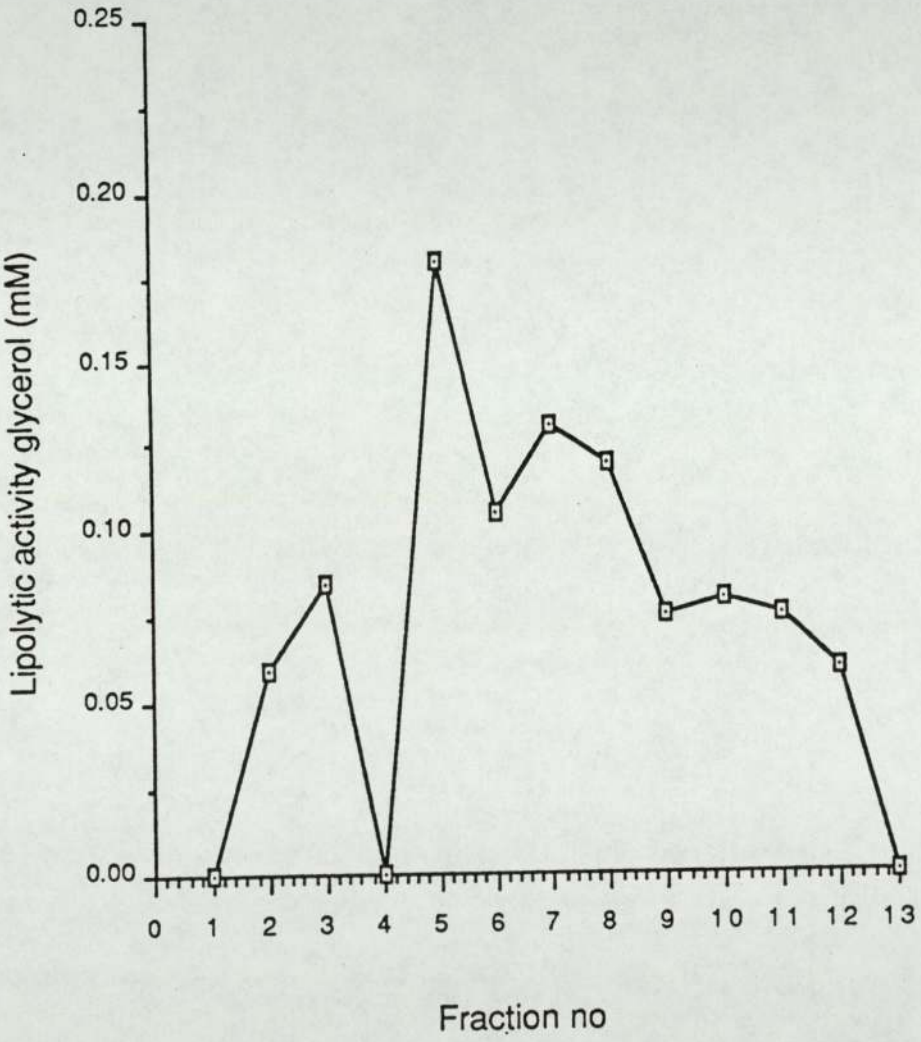
H.P.L.C : C4 rechromatography of peak 1 obtained from purified factor



Lipolytic activity distribution pattern of 0.4ml fractions obtained from a C4 column run on a 30 min gradient of 10 to 60% acetonitrile : H2O/TFA(0.1%). Run at a rate of 0.2ml/min. The three peaks correspond (respectively in order of elution) to molecular weights of 3, 1.5 and 0.8kD as measured by Sephadex G50 chromatography.

Figure 47 (a)

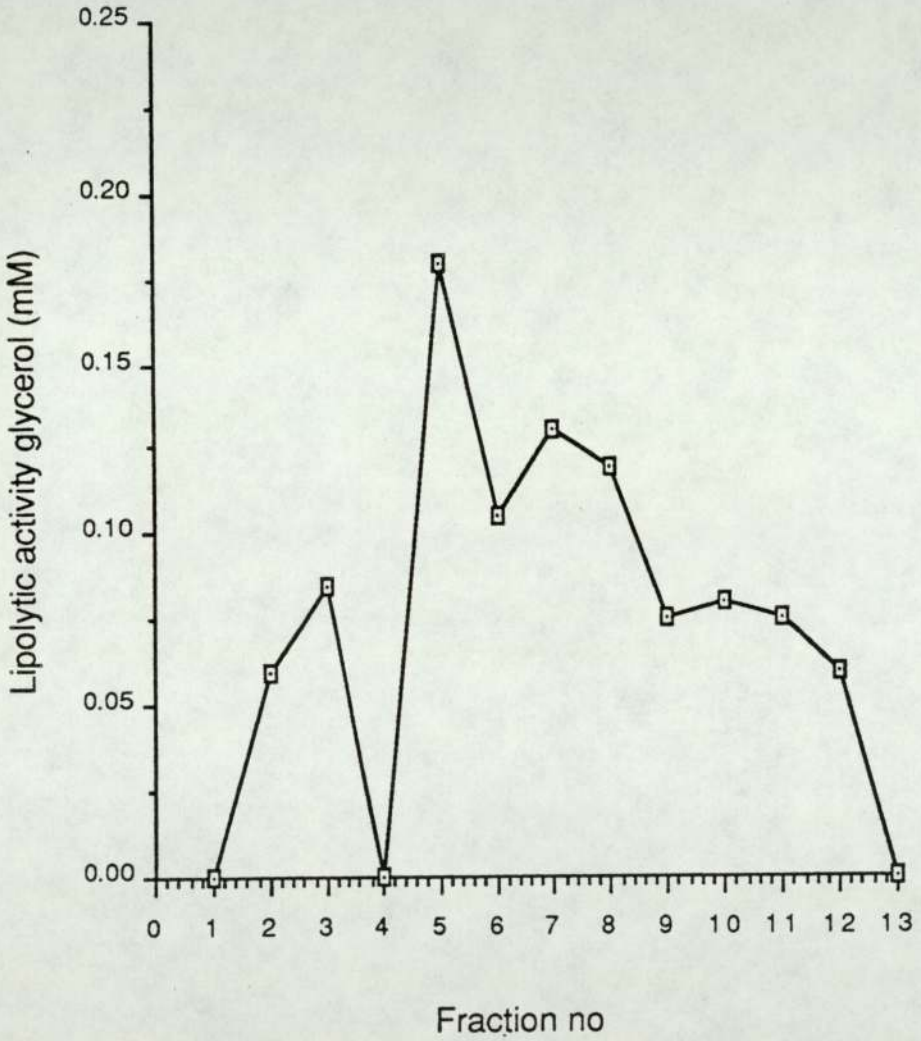
H.P.L.C: P2 biogel chromatography of partially purified lipolytic activity



ipolytic activity distribution pattern of 0.4ml fractions obtained from a
2 column run in 5.0mM sodium phosphate buffer pH8.0. Eluted at a rate
f 0.2ml/min.

Figure 47 (a)

H.P.L.C: P2 biogel chromatography of partially purified lipolytic activity



Lipolytic activity distribution pattern of 0.4ml fractions obtained from a P2 column run in 5.0mM sodium phosphate buffer pH8.0. Eluted at a rate of 0.2ml/min.

Figure 47 (b)

Hplc trace of P2 biogel chromatography.

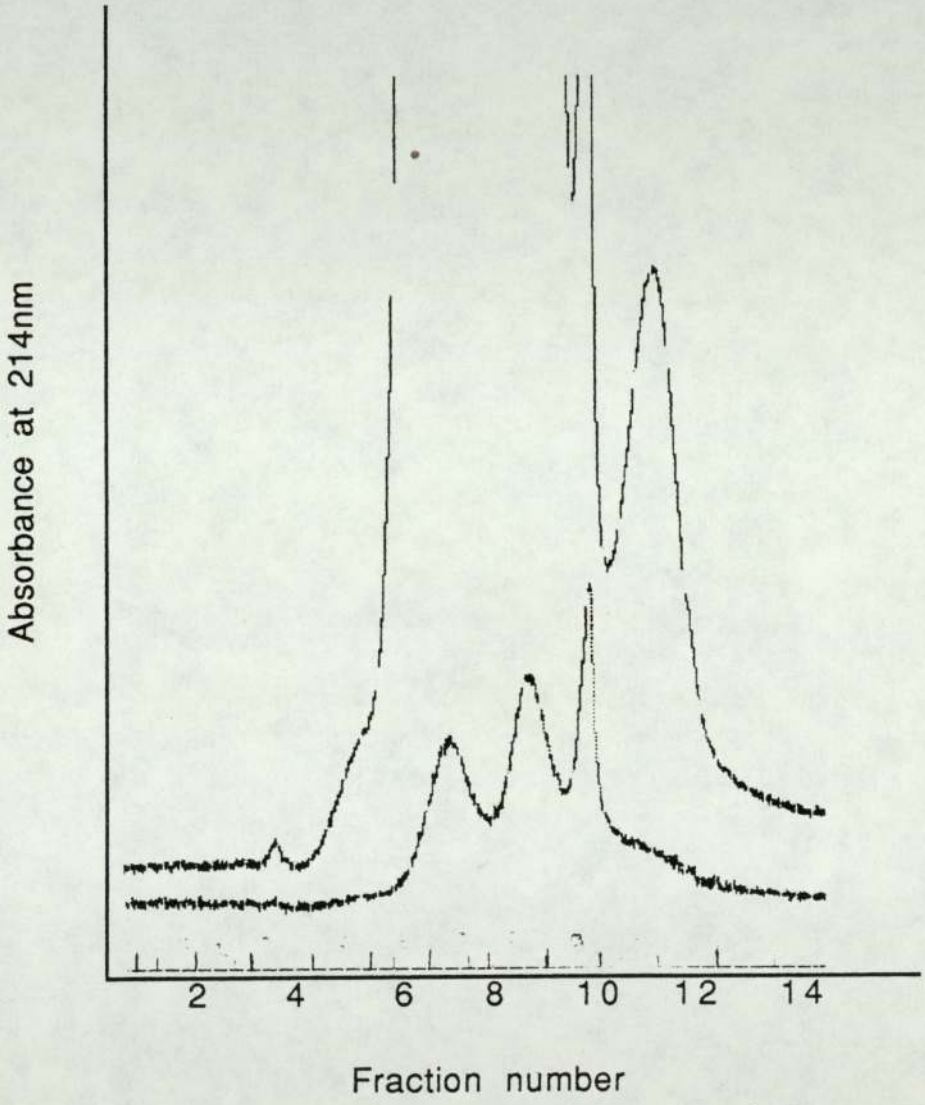
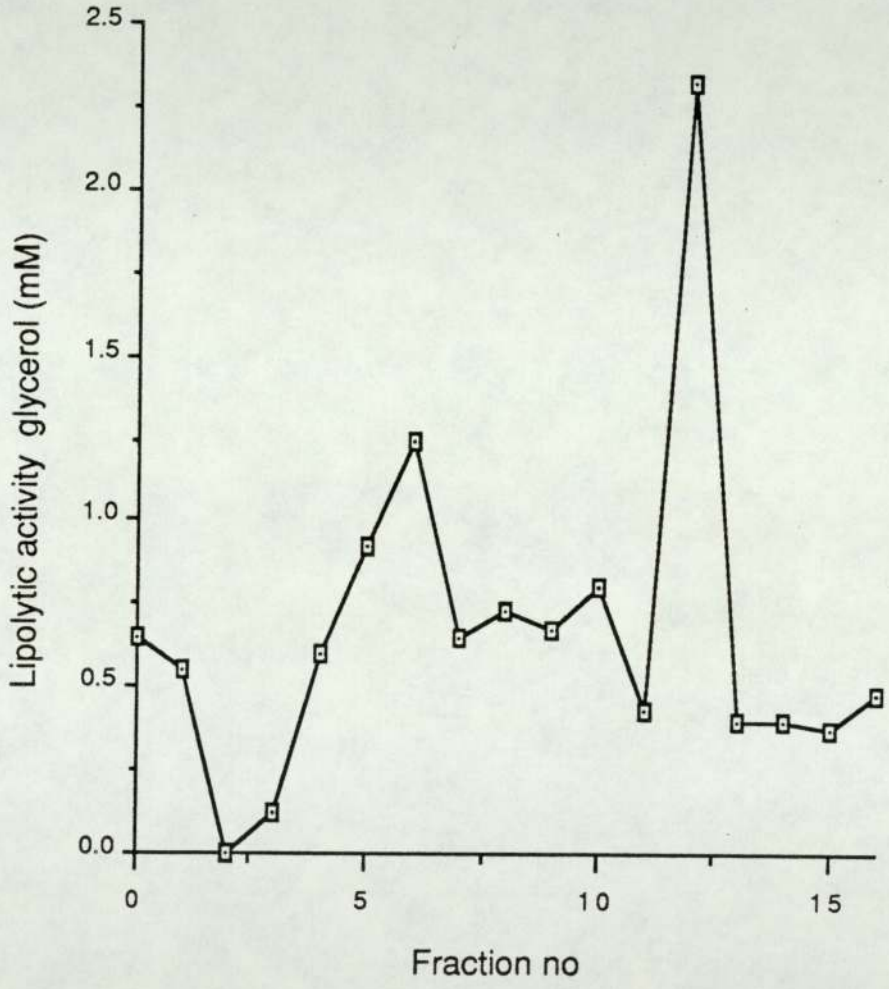


Figure 48 (a)

H.P.L.C: DE anion exchange chromatography of partially purified lipolytic activity



Lipolytic activity distribution pattern of 0.4ml fractions obtained from a DE anion exchange column in 0.01mM phosphate buffer pH8.0 and eluted with a 0 to 0.2m KCl gradient.

Figure 48 (b)

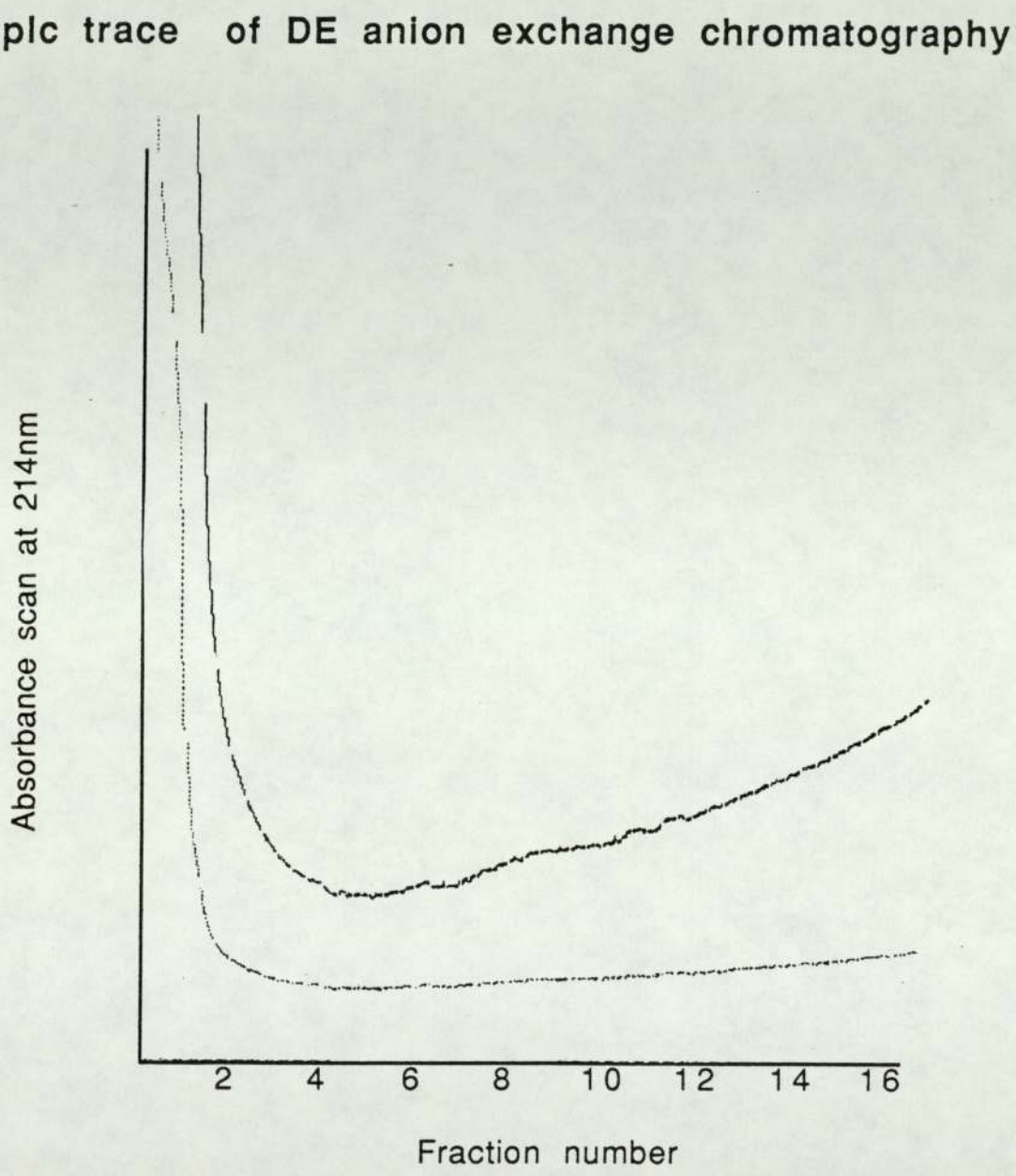
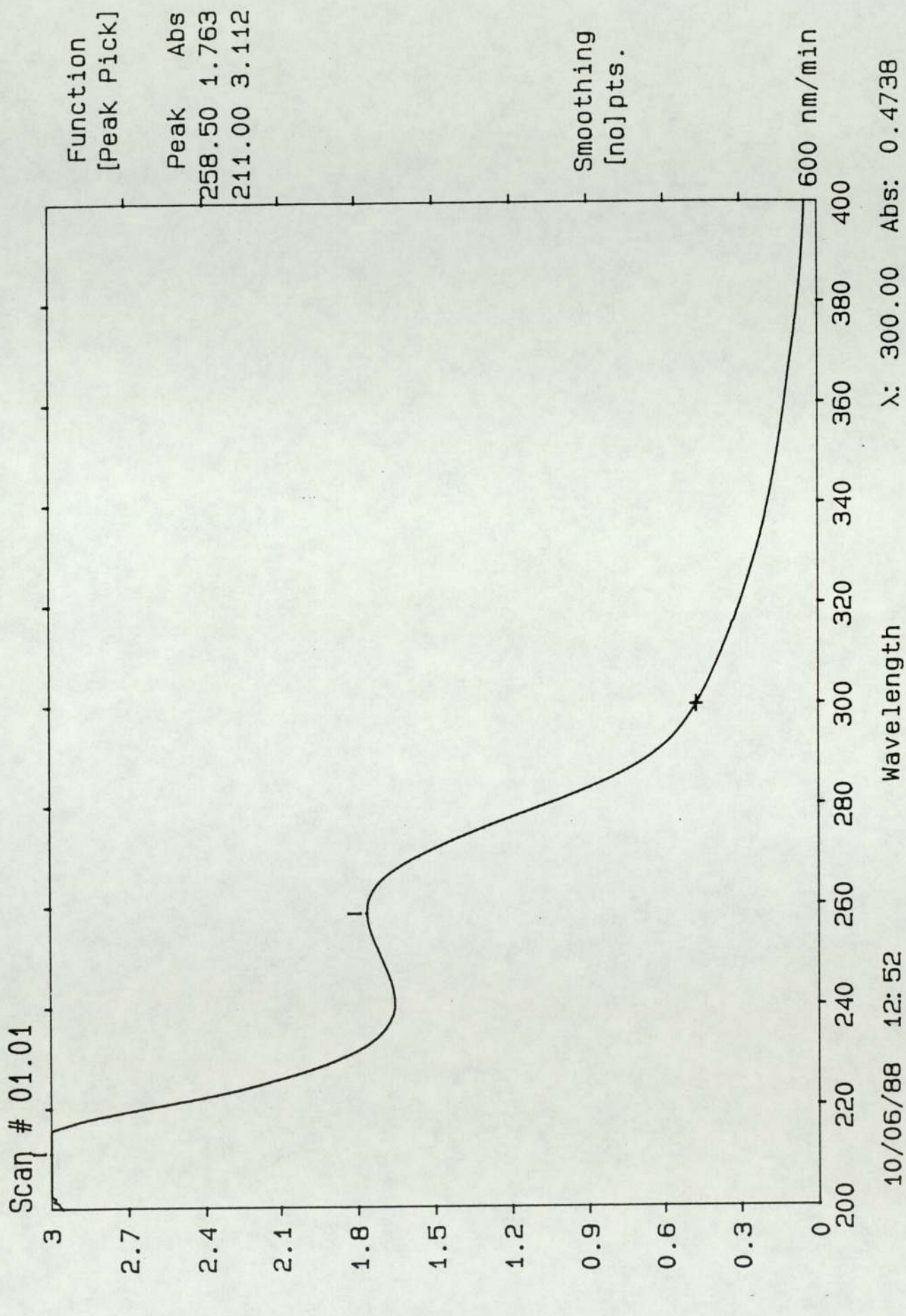

Hplc trace of DE anion exchange chromatography
Absorbance scan at 214nm
2
4
6
8
10
12
14
16
Fraction number

Figure 49


Function
[Peak Pick]
Peak Abs
258.50 1.763
211.00 3.112
Smoothing
[no]pts.
600 nm/min
Scan # 01.01
Absorbance
3
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
200
220
240
260
280
300
320
340
360
380
400
Wavelength
10/06/88 12:52
λ: 300.00 Abs: 0.4738

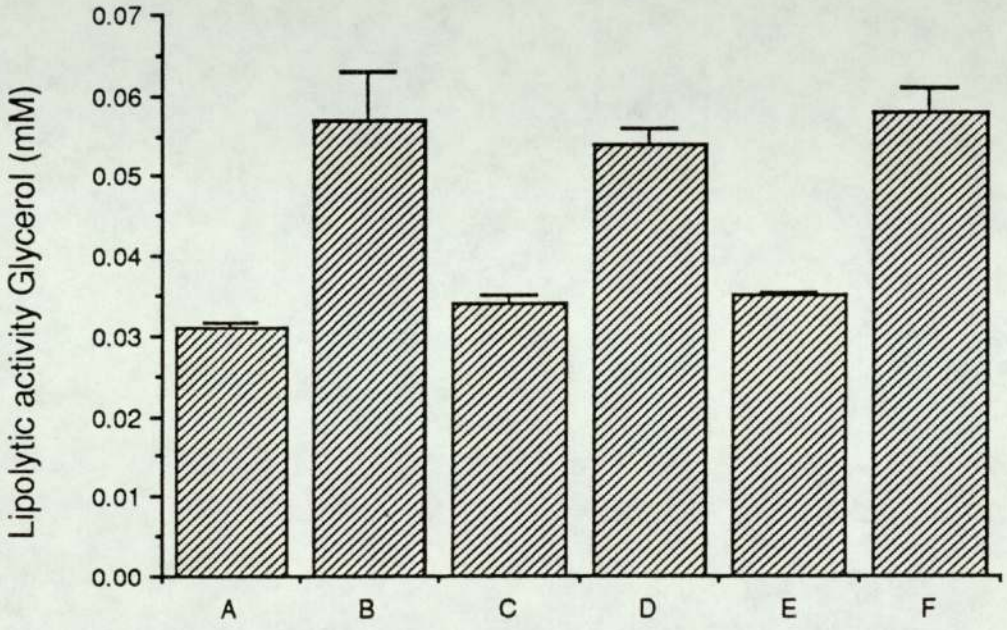
producing a $KClO_4$ precipitate. It is possible that the lipolytic factor produced by the MAC16 tumour adheres to the proteins and was carried down with the precipitate. As we have shown before it seems the lipolytic factor produced by the MAC16 tumour adheres to other proteins easily.

RNAase and DNAase had no effect on the lipolytic activity produced by the factor purified from the MAC16 tumour (fig 50).

Alkaline phosphatase inhibited the lipolytic factor produced by the MAC16 tumour (table 15). However the amount of inhibition seen , is dependent on the concentration of factor and enzyme used. Acid phosphatase and sulphatase had no effect on the lipolytic factor produced by the MAC16 tumour.

Figure 50

Effect of RNAase and DNAase on purified lipolytic factor



A. control basal lipolytic activity
B. 50µl factor
C. RNAase 1.0mg/ml
D. 50µl factor + RNAase 1.0mg/ml
E. DNAase 1.0mg/ml
F. 50µl factor + DNAase 1.0mg/ml

The results are expressed as the Mean ± S.E.M of 3 experiments.

Table 15

Effect of alkaline phosphatase on lipolytic factor produced by the MAC16 tumour.

1. 50µl factor plus 10 units/ml of alkaline phosphatase

Factor alone mM glycerol	Factor +AP mM glycerol	% Inhibition
0.034±0.001	0.012±0.003	64.0

2. 100µl factor plus 20units/ml of alkaline phosphatase

Factor alone mM glycerol	Factor +AP mM glycerol	% Inhibition
0.085±0.001	0.055±0.002	36.0

All incubations were carried out for 20 hours at 37°C. The results are expressed as the Mean ± S.E.M of 3 experiments. Controls of adipocytes alone and alkaline phosphatase plus adipocytes were subtracted from the appropriate samples.

4.4.3 Discussion

The MAC16 adenocarcinoma appears to produce more than one lipolytic and proteolytic factor. These factors are both acidic, are distinguishable by molecular weight and by the total charge on each factor. The proteolytic and lipolytic factors are seperable by Sephadex chromatography; the proteolytic factor being of molecular weight >150k daltons and the lipolytic factors of molecular weight of approximately 3k, 1.5k and 700 daltons. The same lipolytic factors are found, although to a lesser extent, in the MAC13 adenocarcinoma, which does not confer cachexia on its host. These factors may be required for tumour growth but an over expression of these factors causes cachexia. Further purification of the lipolytic factors was carried out using reverse phase hplc. The lipolytic factor seems to be a very potent substance. The concentration needed to show activity in the bioassay perhaps in the order of nM or pM. Thus no peaks of absorption are detected on the hplc traces as of yet. From the absorbance scan a peak at 260nm can either imply a nucleic acid or a hydrophobic amino acid like phenylalanine or tryptophan. 211nm (214nm) is a standard peak indicating the presence of amino acids. Further experiments using the purified material showed the factor was not an oligosaccharide since it was not destroyed by periodate. The factor was not destroyed by heat, but as it is such a small peptide it is unlikely that heat would break any important bonds and so reduce activity. This is in contrast to the tumour extract discussed earlier in section 4.2, where it is possible that the peptide sticks to larger

proteins and is precipitated out by the heat.

The partially purified factor produced by the MAC16 tumour was acid stable and not degraded by RNAase and DNAase. Thus the factor may be a peptide rather than a nucleotide. However the factor may be a small oligonucleotide that is not affected by these nucleic acid degrading enzymes.

Isoelectric focussing of the lipolytic factor produced by the MAC16 tumour was carried out at the Middlesex hospital, University of London. The isoelectric point of the factor was <1.0 indicating the presence of a highly acidic group such as a sulphate or phosphate group on the molecule. Sulphatase had no effect on the lipolytic activity produced by the factor from the MAC16 tumour. A reduction in the lipolytic activity of the factor produced by the MAC16 tumour was observed with alkaline phosphatase. This indicates the possible presence of a phosphate group in the sequence of the factor. However the alkaline phosphatase did not completely abolish the lipolytic activity of this tumour factor. Thus the phosphate group may not be essential for its activity or it may not be a very good substrate for alkaline phosphatase. Therefore the factor may be a phosphorylated peptide perhaps with a serine phosphate in its sequence. This can only be determined after peptide sequencing and amino acid analysis has been carried out.

The lipolytic factor was acid stable ,heat stable and partially degraded by pronase (1.0mg/ml) which may indicate a peptidic nature. To fully elucidate the structure of

these lipolytic factors amino acid analysis and protein sequencing is required. After completion of this the lipolytic material could be synthesised, monoclonal antibodies prepared and used in the treatment of cachexia.

Identification of the lipolytic factors produced by the MAC16 adenocarcinoma in other tumour models and body fluids of mice and man are reported in the next section.

4.5 Identification of lipolytic factors present in a human tumour model and in the body fluids of mice and man.

4.5.1 Introduction

Enhanced lipolytic activity has been found in tumour models other than the MAC16 adenocarcinoma and in the body fluids of both mice and humans (table 7,8,9,10 and 11). Further purification of lipolytic factors present in these tissues was undertaken to compare and contrast with factors found in the MAC16 adenocarcinoma (section 4.4). In addition the presence of these factors in other diseases associated with weight loss was investigated.

Alzheimers disease is a disease of the brain contracted in old age. It is the commonest type of dementia. This syndrome is caused by an overproduction of fibrous proteins in the neurones of the brain. As a result abnormally formed microtubles and neurofilaments pack the cells to the exclusion of other vital subcellular organelles and the cells die (W.C. Bowman and M.J. Rand, Textbook of Pharmacology). In this disease many patients lose large amounts of weight with no apparent reduction in food intake (Singh et al 1988). The group we have studied lost between 6.4 - 37.1 kg over a period of up to 18 months. Nutritional assessments were performed in 3 groups of hospitalised elderly women comparable in age and mobility. The three groups consist of 1. Alzheimers patients, 2. Multi-infarct demented patients and 3. Non demented patients. All Alzheimer patients lost weight and on average weighed 21% less than nondemented patients and 14% less than

multi-infarct demented patients. The weight loss was not accounted for by any defecit in food intake or malabsorption. It was concluded that the reason for weight loss in Alzheimers patients remains unknown (Singh et al 1988). Significant elevation of cortisol secretion in elderly depressives has been reported in Alzheimer patients. Whether this causes the weight loss noted remains to be determined (Ferrier et al 1988). It has been reported that patients suffering from Downs syndrome who develop Alzheimers disease from the age of fourty also suffer from weight loss (Heston, 1977). Albalon et al (1984) put forward the hypothesis that the etiology of Alzheimers disease is malnutrition. He states that "the predominant deficiency of one or more nutrients could explain the predominamce of certain symptoms in a patient". Nutritional treatment is of great value to Alzheimer patients they live longer and it prevents complications of malnutrition like infectious diseases.

Enhanced lipolytic activity was also found in patients suffering from multiple burns. Patients with burns are known to lose a lot of weight partially due to water loss. However the loss in weight may in part be a result of the enhanced lipolytic activity. Similar to trauma induced cachexia following a burns injury patients have a hypermetabolic rate and considerable weight loss is experienced although patients are eating normally (Mason, 1979). This hypermetabolism begins approximately a day after the injury and its level increases for a week until it reaches a plateau. This plateau is maintained for 10

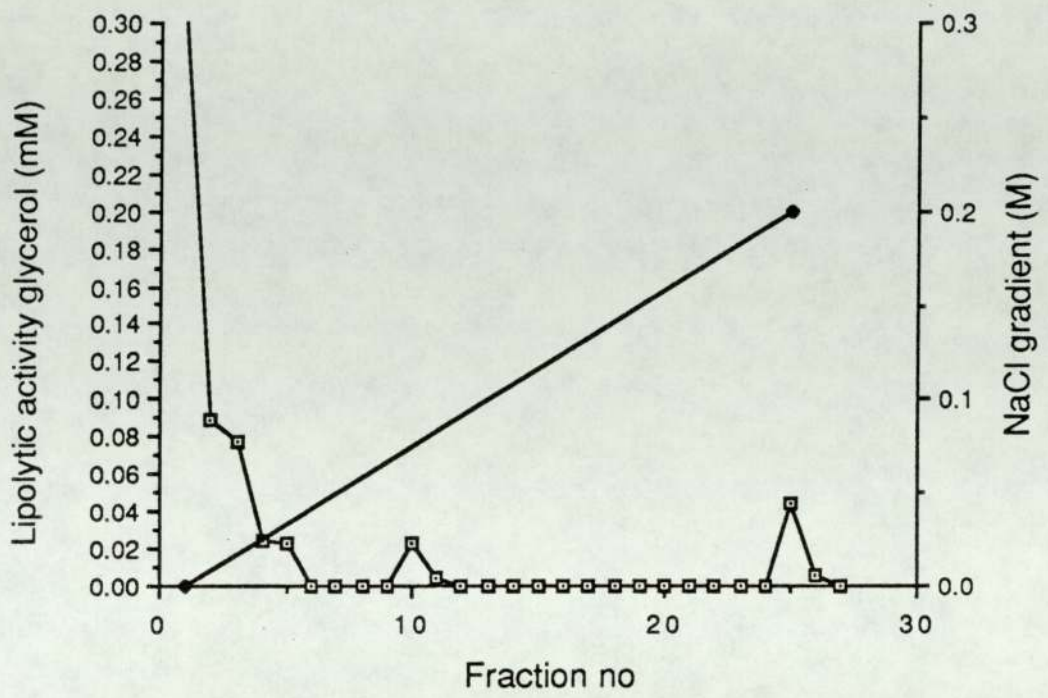
days to 2 weeks and then returns to normal as the patient heals. Energy expenditure as much as twice normal has been documented during the first 3 weeks after a burn injury (Newsome, 1973). Therefore during this hypermetabolic state caloric requirements are increased, failure to provide extra calories will result in a dramatic weight loss (Curreri, 1978).

4.5.2 Results

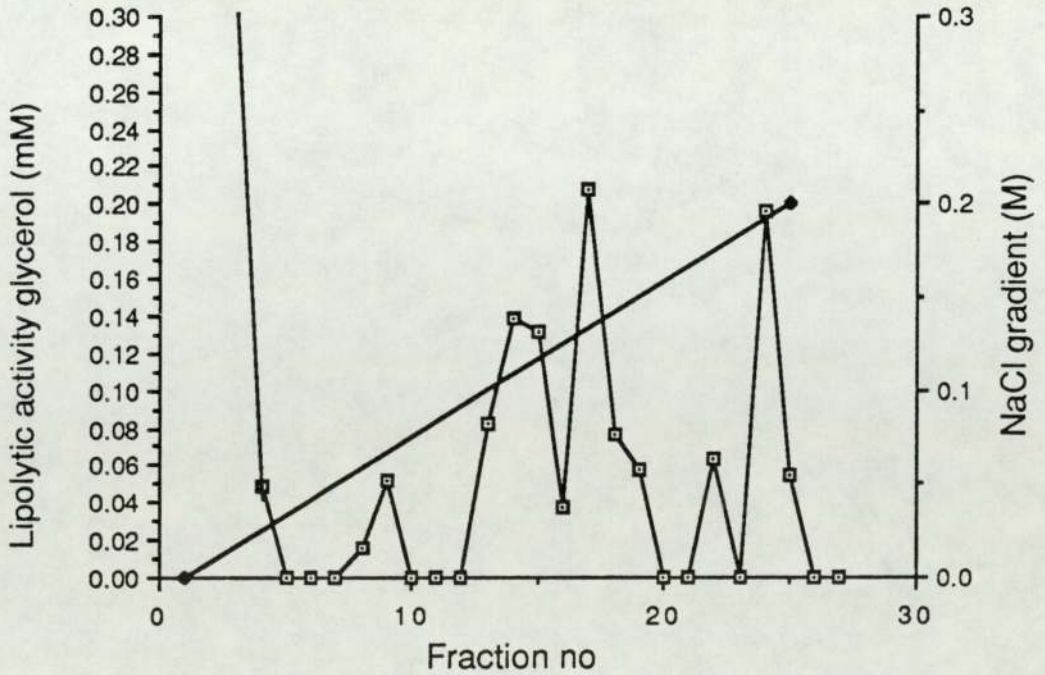
Serum sanples from MAC16 tumour bearing mice have shown high lipolytic activity but there was not enough measurable activity detectable after DEAE cellulose chromatography. DEAE cellulose chromatography was performed on two serum samples from cancer patients (fig 51 and 53). The first patient (A.M) had a lymphoma and had lost 19.6 kg at the time serum was taken. The second patient (A.H) had a bladder neoplasm and had lost 9.0 kg at the time serum was taken. For comparison two serum samples from non-tumour bearing healthy individuals were passed down a DEAE cellulose column (fig 51 and 53). The control subjects showed a large peak not retained by the DEAE column. This was most likely due to the presence of normal lipolytic hormones in the serum which are all basic and would not be retained by a DEAE column . The cancer patients showed this peak but also gave a similar profile to that seen in the MAC16 adenocarcinoma (fig 52). The active peaks (excluding the initial peak) from patient A.H were subjected to Sephadex G50 column chromatography to define the molecular weights of this material. Patient A.H showed active peaks

Figure 51

DEAE cellulose chromatography of a control serum sample(1.0ml) from a non-tumour bearing individual(N.H)

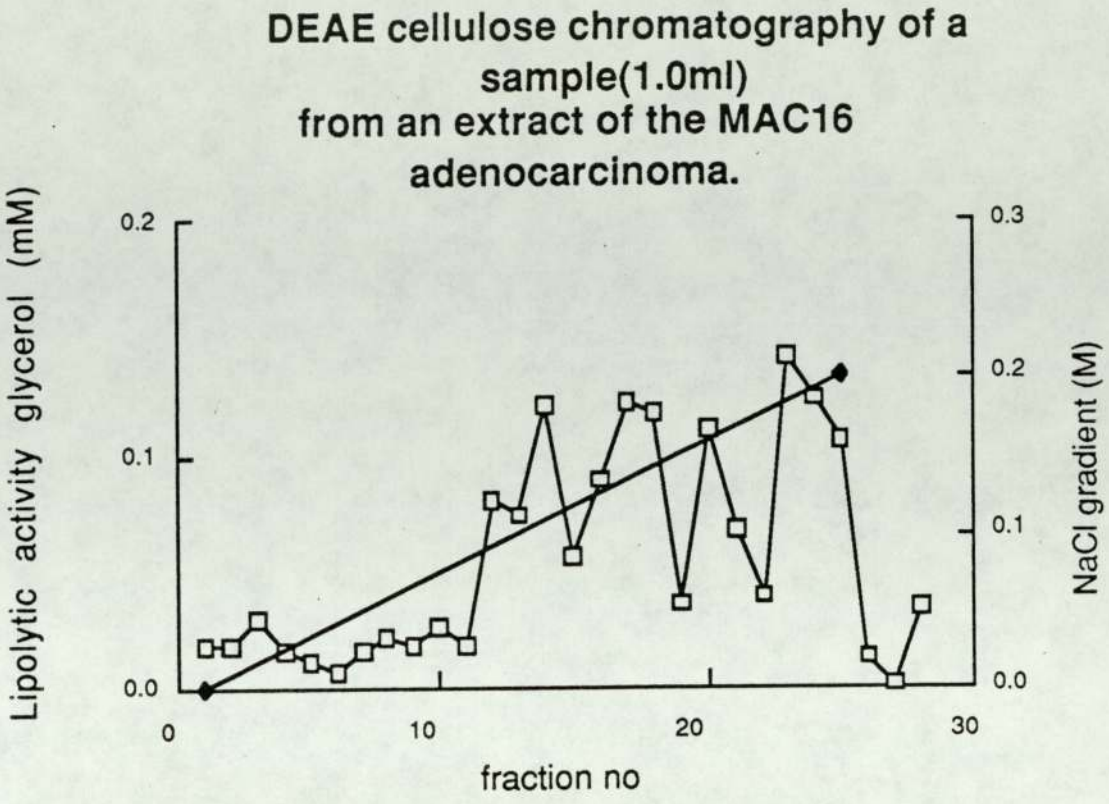


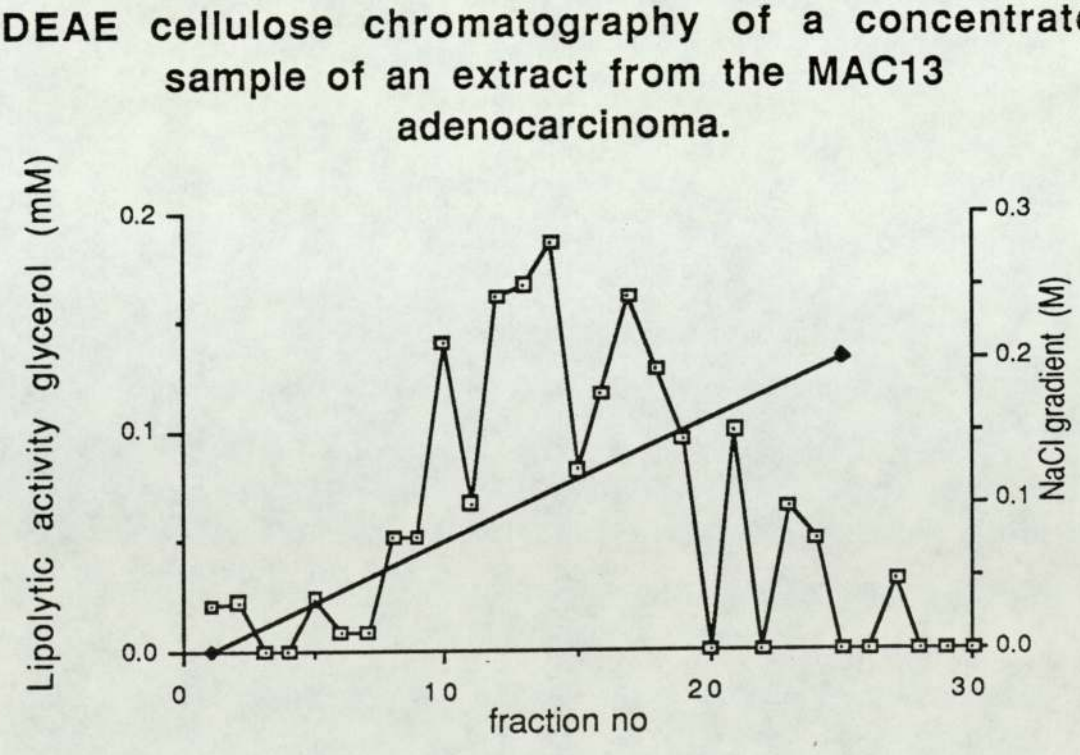
DEAE cellulose chromatography of a sample(1.0ml) of serum from a cancer patient(A.M)



Lipolytic activity distribution pattern of 1.0ml fractions obtained from a DEAE column in phosphate buffer pH8.0 eluted with a 0-0.2M NaCl gradient.

Figure 52

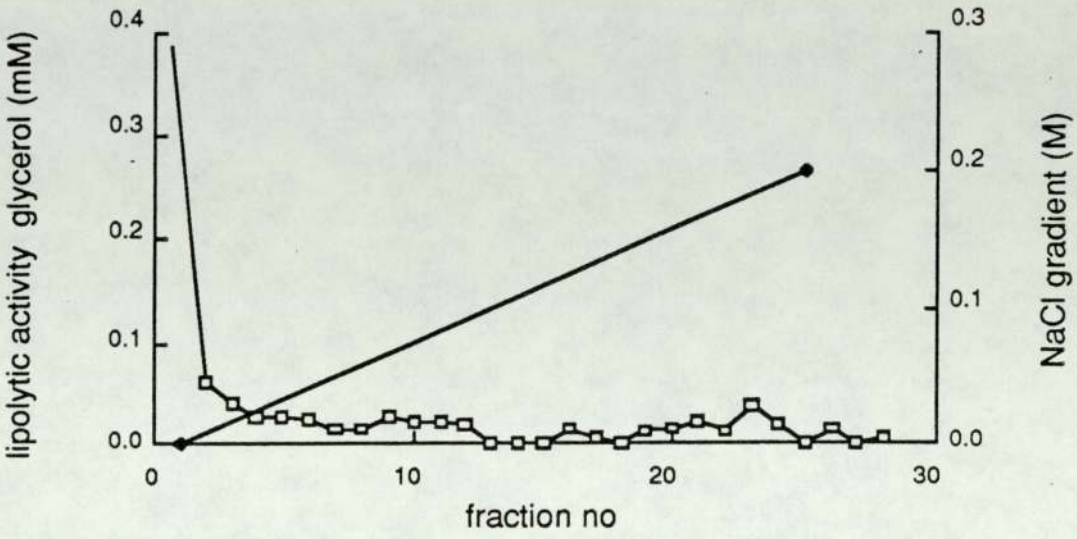




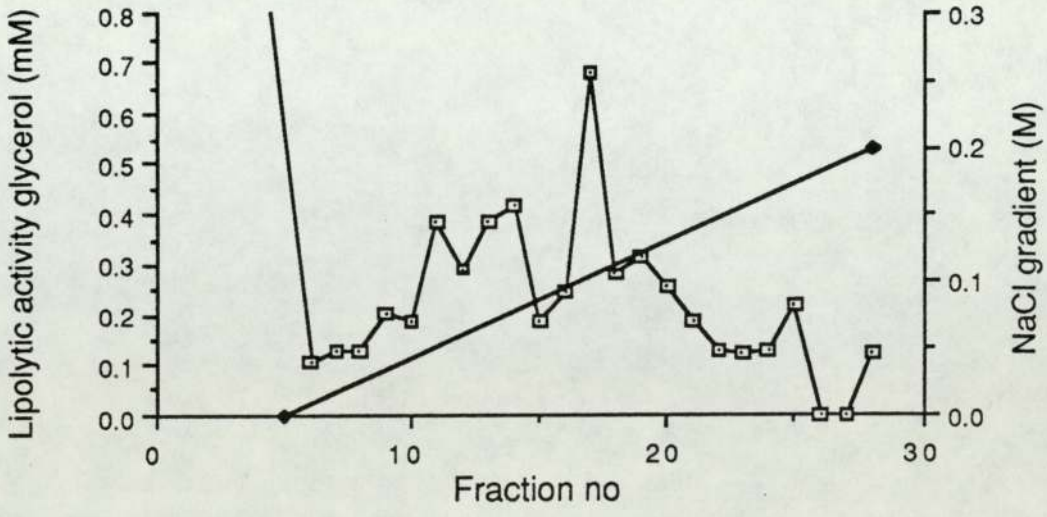
Lipolytic activity distribution pattern of 1.0ml fractions obtained from a DEAE column in phosphate buffer pH 8.0 eluted with a 0-0.2M NaCl gradient.

Figure 53

DEAE cellulose chromatography of a control serum sample from a non-tumour bearing individual (A.S).



DEAE cellulose chromatography of a serum sample (1.oml) from a cancer patient (A.H)



Lipolytic activity distribution pattern of 1.0ml fractions obtained from a DEAE column in phosphate buffer pH8.0 run on a 0-0.1M NaCl gradient.

of the same molecular weights as seen in the MAC16 adenocarcinoma namely 3, 1.5, and 0.7k daltons (fig 54).

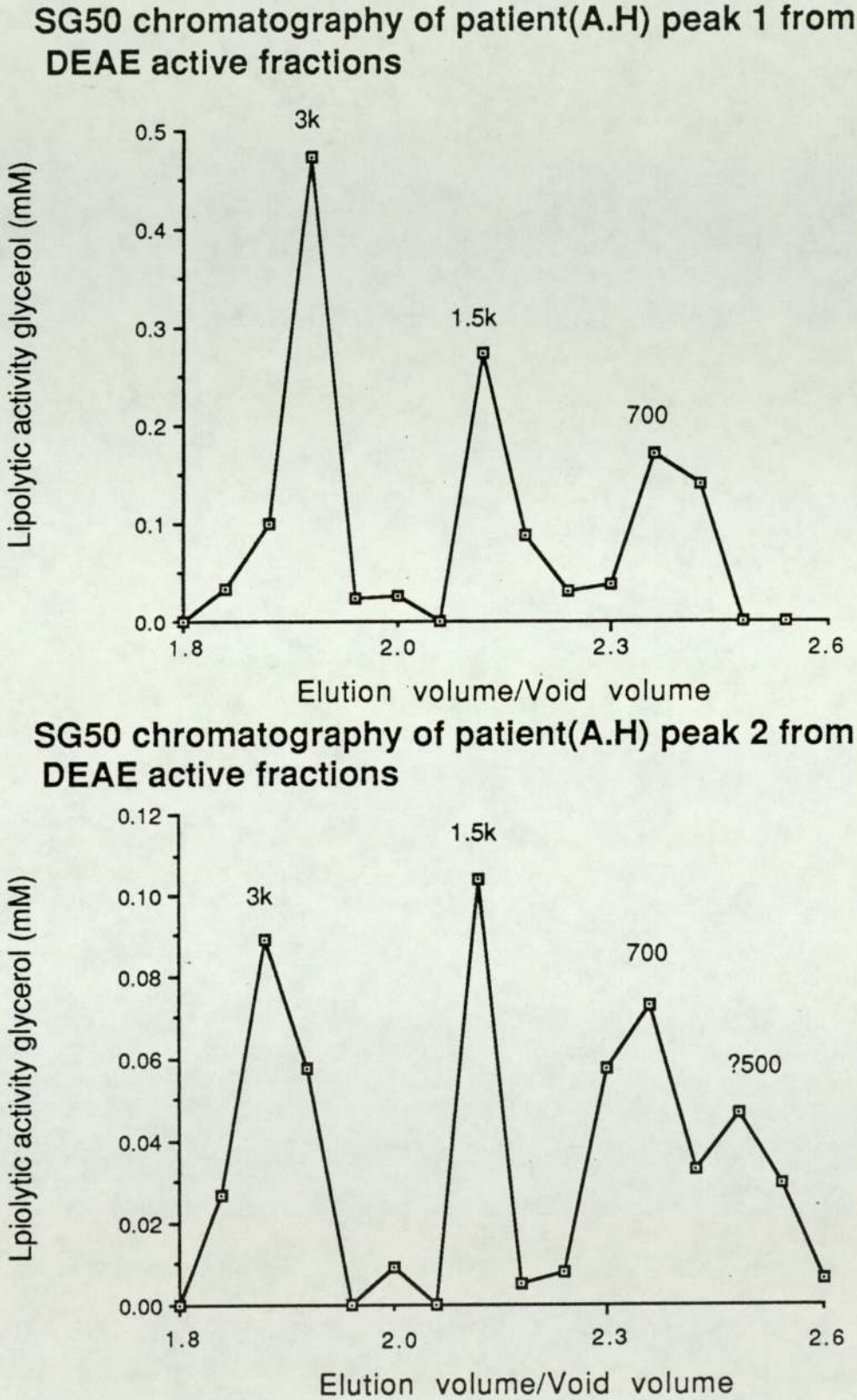
Serum from a patient (D.C) suffering from multiple burns when passed down a DEAE cellulose column expressed low activity in the region where the lipolytic factors from the MAC16 adenocarcinomas were seen (fig 55). More serum from this patient was rechromatographed on a Sephadex G50 column and many active peaks were found. However there was no active peaks in the area of molecular weight range 3.0 to 0.7k daltons. The peaks seen were of larger molecular weights (fig 56).

Sera from Alzheimer patients that expressed some lipolytic activity were pooled and 28.0ml of this pooled serum was then lyophilized, redissolved and passed down a Sephadex G50 column (fig 57). No active peaks in the region 3 to 0.7kD were seen.

As described in section 4.3 starvation causes an increase in lipolytic activity in the serum of non tumour bearing individuals. A 1.0ml serum sample was taken from a non tumour bearing healthy individual (A.S) after a 24hr starvation period, and this was subjected to Sephadex G50 chromatography. No peaks of lipolytic material were found in the molecular weight range when compared to a weight losing cancer patient (fig 58).

Urine samples fromn tumour bearing individuals also have an enhanced lipolytic activity (section 4.3). An aliquot of urine (1.0ml) from a mouse bearing the MAC16 tumour was chromatographed on a Sephadex G50 column. A large peak of activity of apparent molecular weight of >20k

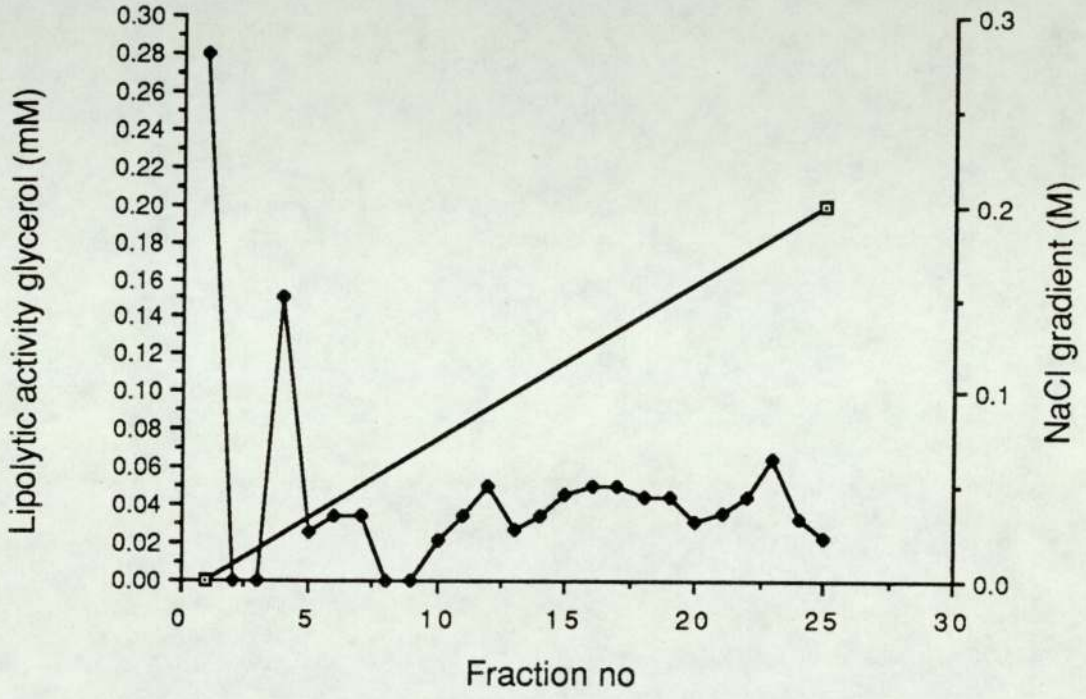
Figure 54



Lipolytic activity distribution pattern of 1.0ml fractions obtained from a Sephadex column eluted with 0.01M phosphate buffer pH8.0.
Void volume= 17.0 ml.

Figure 55

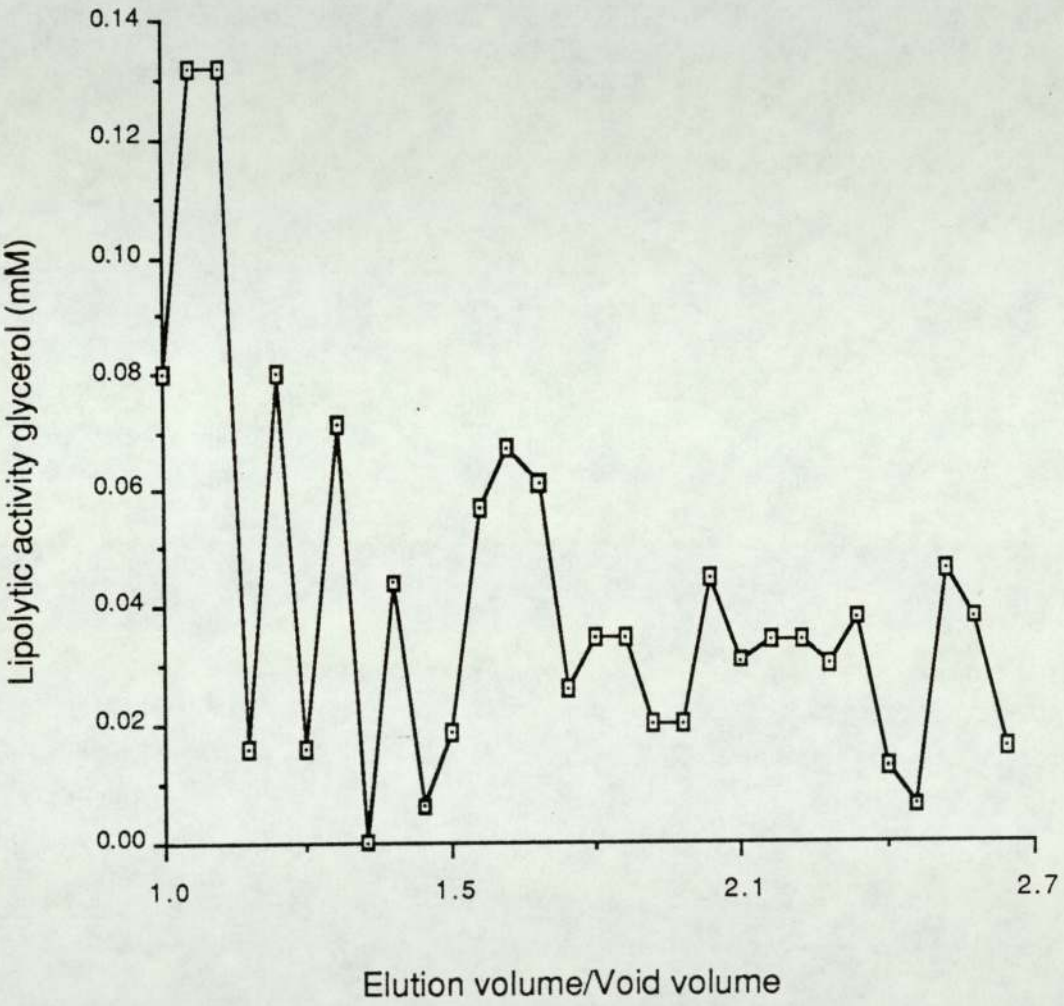
DEAE cellulose chromatography of a serum sample(1.0ml) from a burns patient (D.C).



Lipolytic activity distribution pattern of 1.0ml fractions obtained from a DEAE column in phosphate buffer pH8.0 ran on a 0-0.2M NaCl gradient.

Figure 56

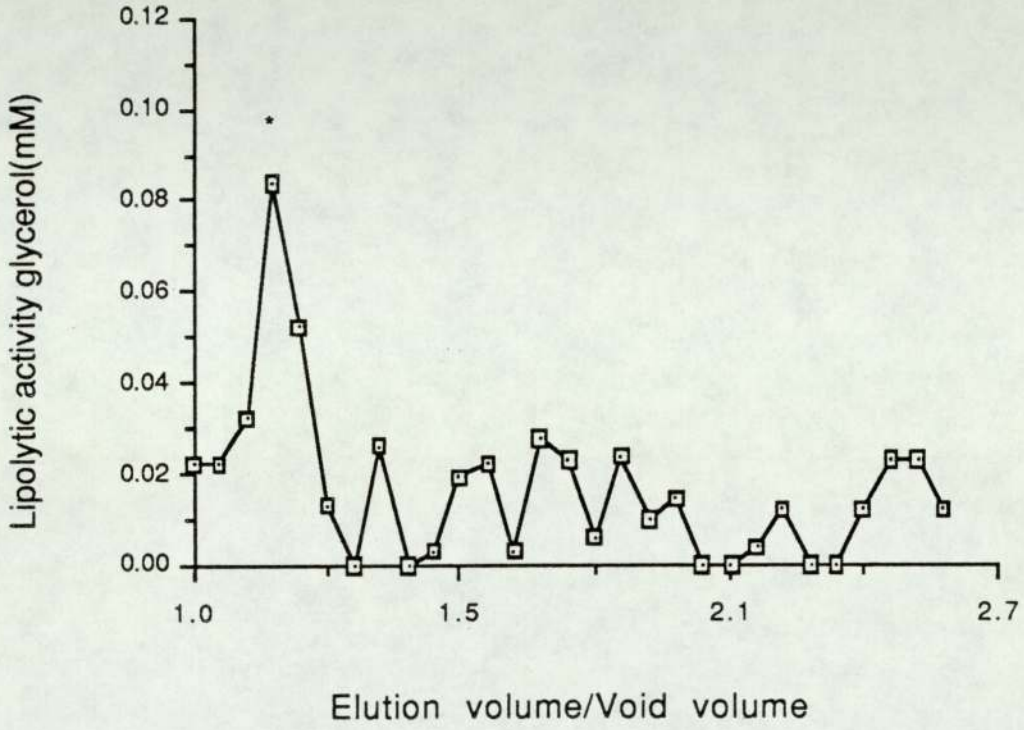
SG50 chromatography of a serum sample (1.0ml) from a patient with multiple burns



Distribution pattern of 1.0ml fractions from a G50 Sephadex column eluted with 0.01M phosphate buffer pH 8.0. Void volume = 17.0ml.

Figure 57

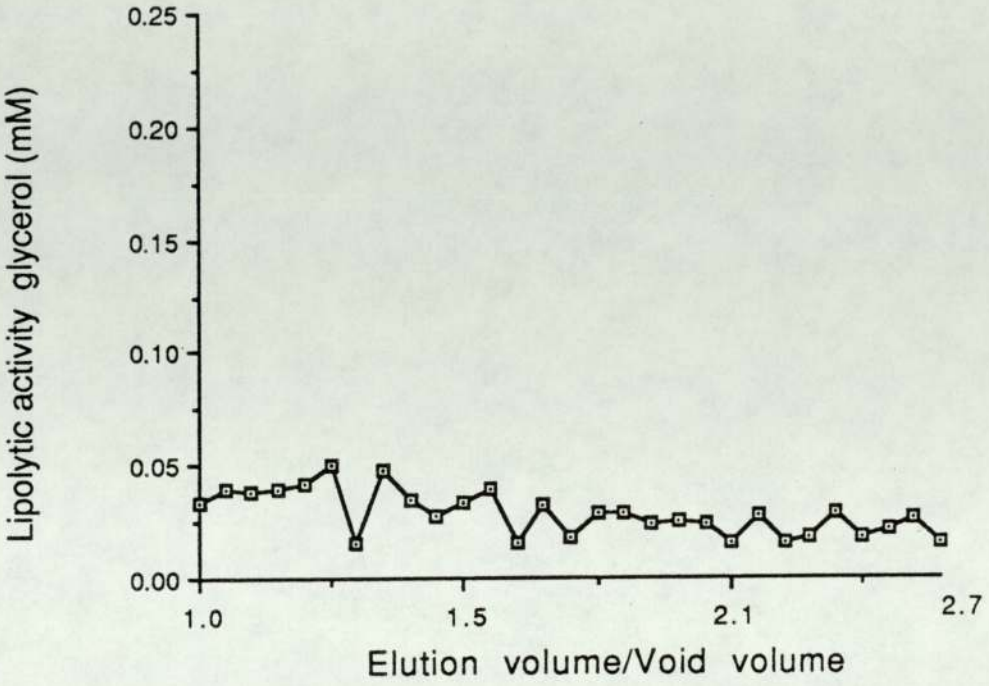
SG50 chromatography of a sample(28.0ml) of serum from Alzheimer patients with weight loss.



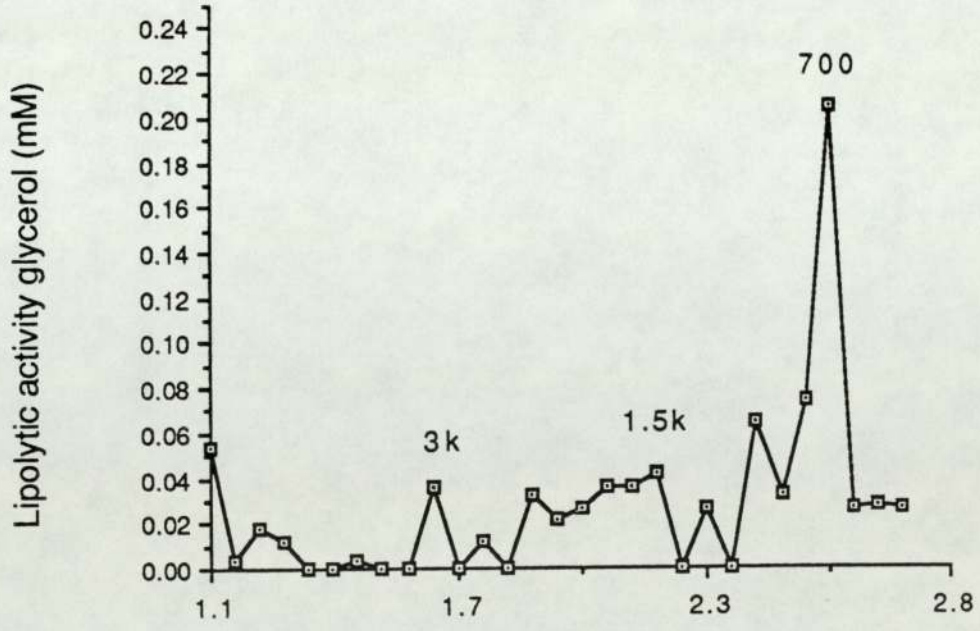
Lipolytic activity distribution pattern of 1.0ml fractions obtained from a Sephadex G50 column ran in phosphate buffer pH8.0. Void volume= 18.0 ml.

Figure 58

SG50 chromatography of a sample(1.0ml) of serum from a control subject(A.S) after 24hr starvation.



SG50 chromatography of a sample(1.0ml) of serum from a cancer patient(I.J)

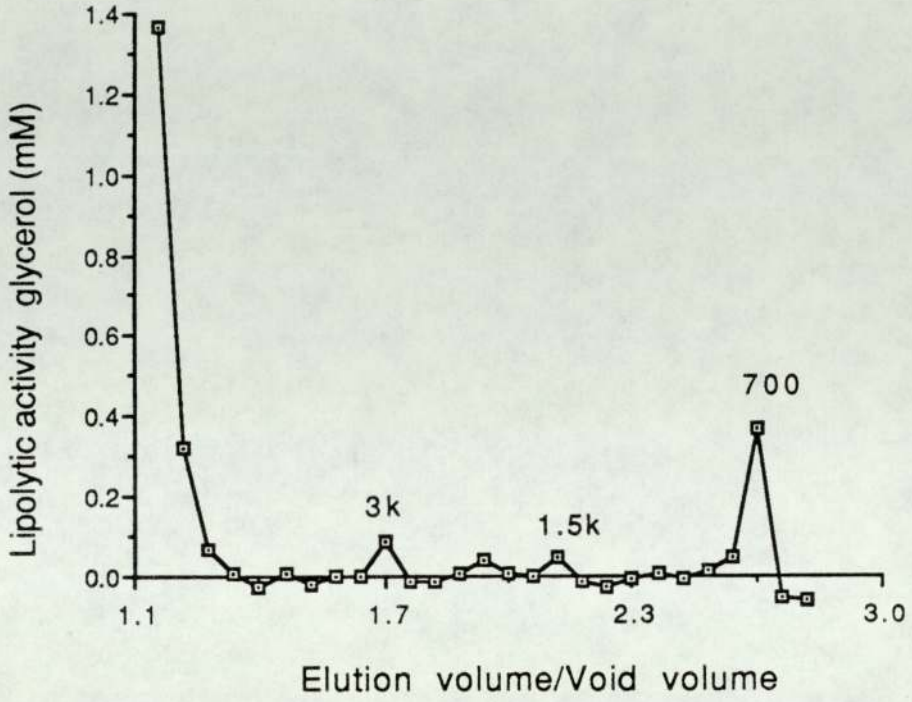


Lipolytic activity distribution pattern of 1.0ml fractions obtained from a G50 sephadex column ran in phosphate buffer pH8.0. Void volume=17.0 ml.

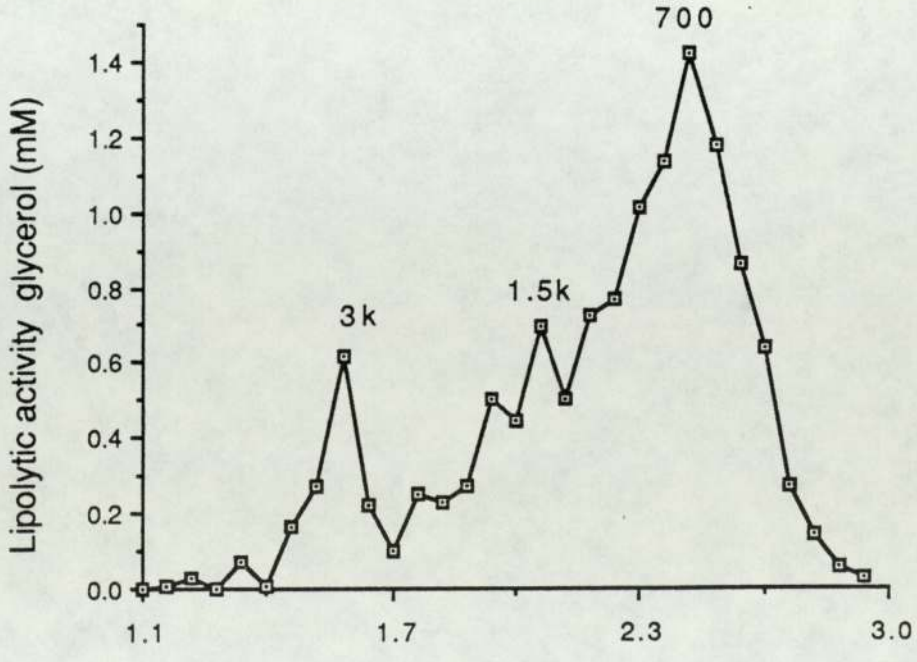
daltons was observed together with smaller peaks of activity as identified earlier of molecular weight 3, 1.5, and 0.7k daltons respectively (fig 59). The larger peak of molecular weight >20k daltons was made up to a 0.5M NaCl solution and rechromatographed on the SG50 column (fig 59). The resulting chromatograph showed a broad range of activity with activity eluting between apparent molecular weights of 3 and 0.7k daltons. In comparison urine from a non tumour bearing mouse was also passed down a Sephadex G50 column and showed no comparable peaks of activity (fig 60). However urine from a mouse bearing the MAC13 tumour when passed down a Sephadex G50 column showed 2 peaks of lipolytic activity of apparent molecular weight 1.5 and 0.7k daltons (fig 60). Urine samples from cancer patients were also collected and examined. Sephadex G50 chromatography was performed on a 1.0ml urine sample from a patient with cervical carcinoma (fig 61). This patient had not lost any weight at the time of collection but still had detectable lipolytic factors in the urine of the same apparent molecular weight as that seen with the MAC16 adenocarcinoma. This was compared to the Sephadex G50 chromatography of a urine sample from a control healthy subject (A.S) (fig 61). The chromatograms of lipolytic activity in the urine of two further cancer patients is shown in fig 62. Patient R had a teratoma and had lost 6.3 kg at the time urine was collected while patient P had a lung carcinoma and had lost no weight at the time urine was collected. Therefore it seems that the same fractions of lipolytic activity are present in all cancer patients with or without weight loss, but the amount is

Figure 59

SG50 chromatography of a sample(1.0ml) of MAC16 urine

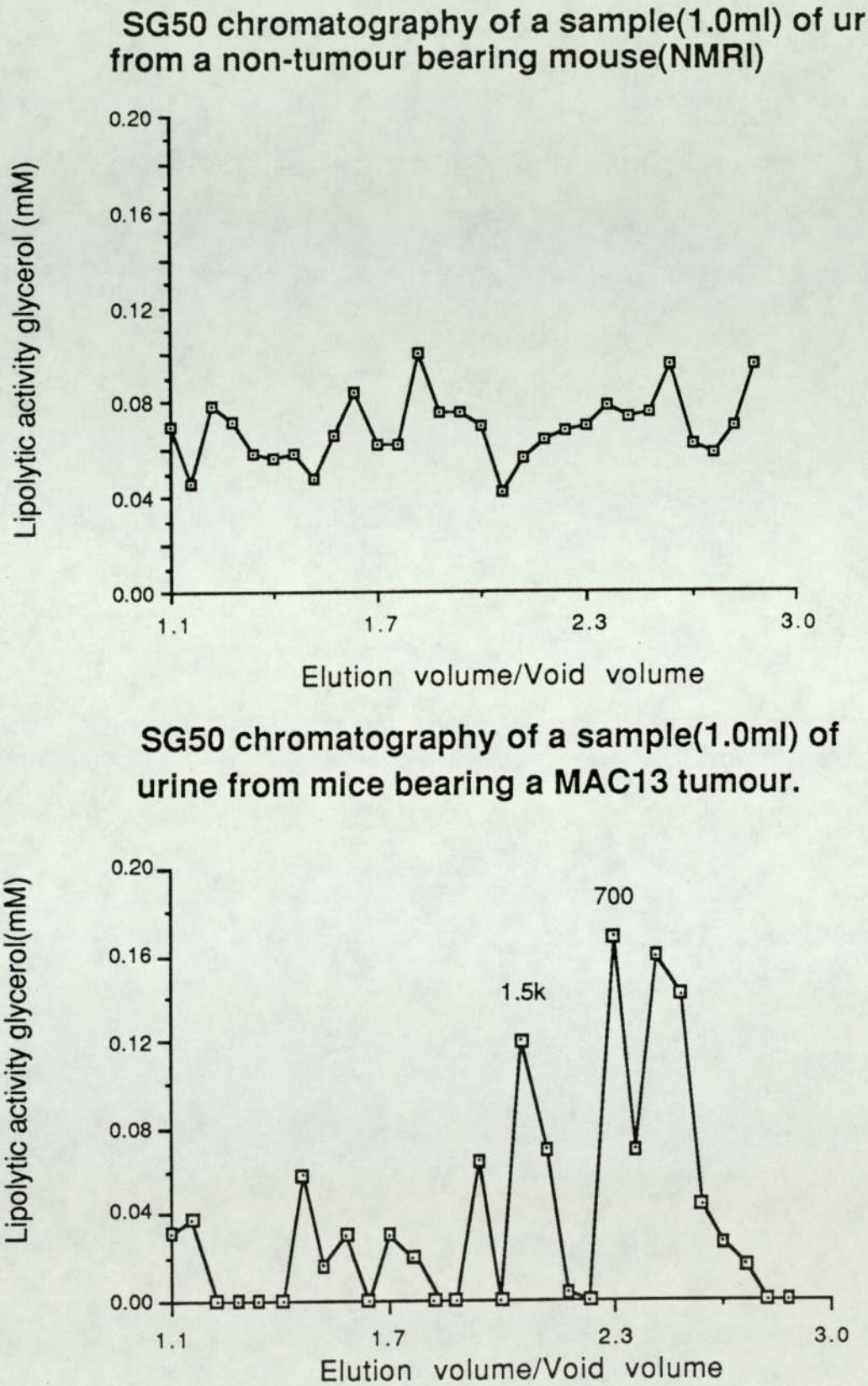


SG50 chromatography of Fraction 17(high mwt sp) from MAC16 urine



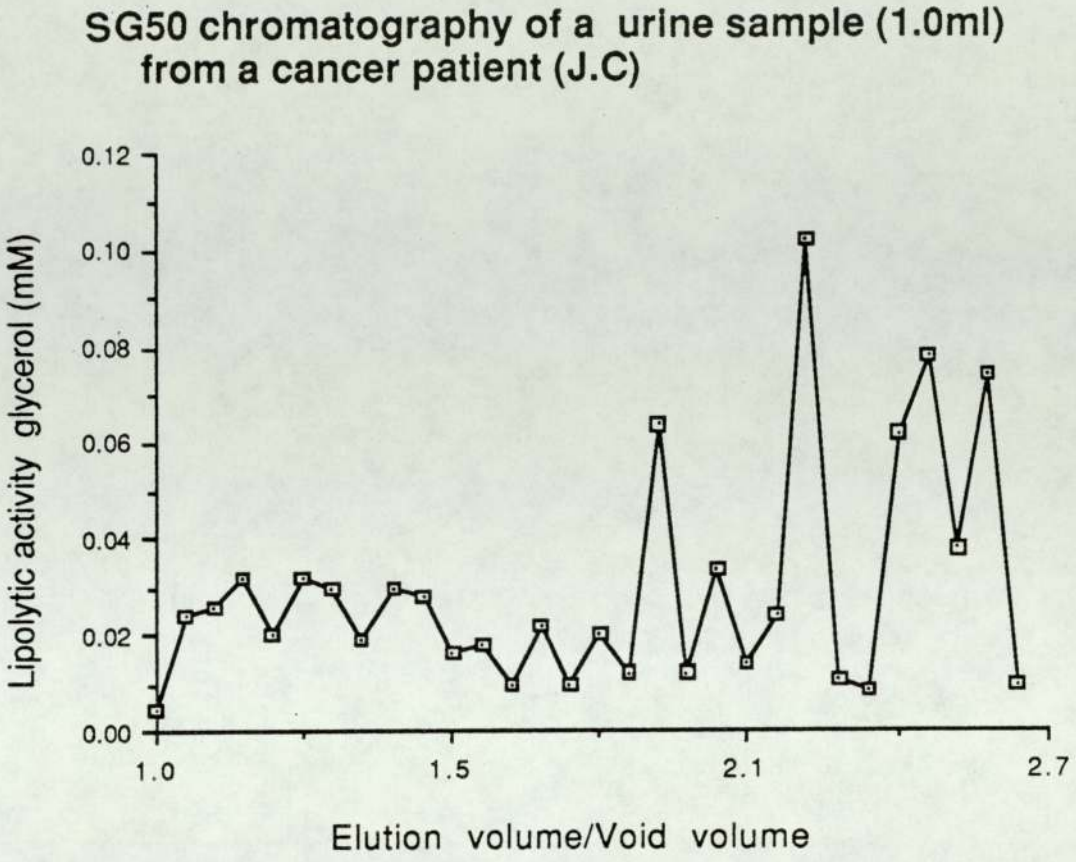
Void volume= 16.0 ml.

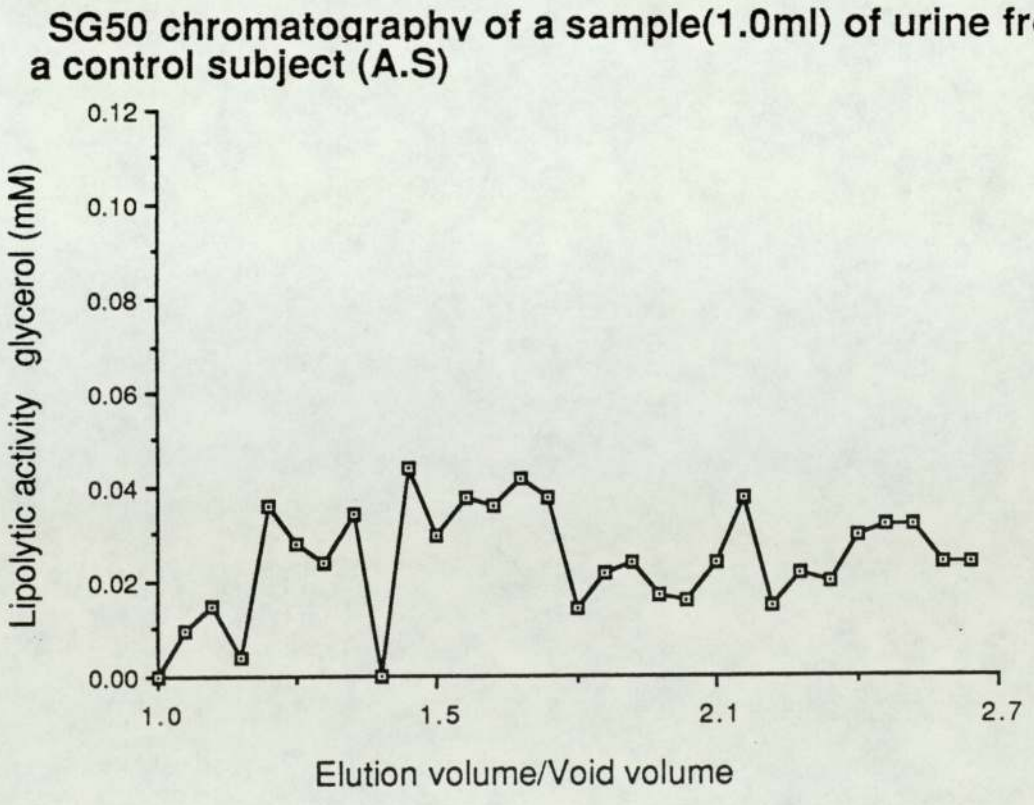
Figure 60



Lipolytic activity distribution pattern of 1.0ml fractions obtained from a Sephadex G50 column eluted with 0.01M phosphate buffer | Void volume= 18.0 ml.

Figure 61

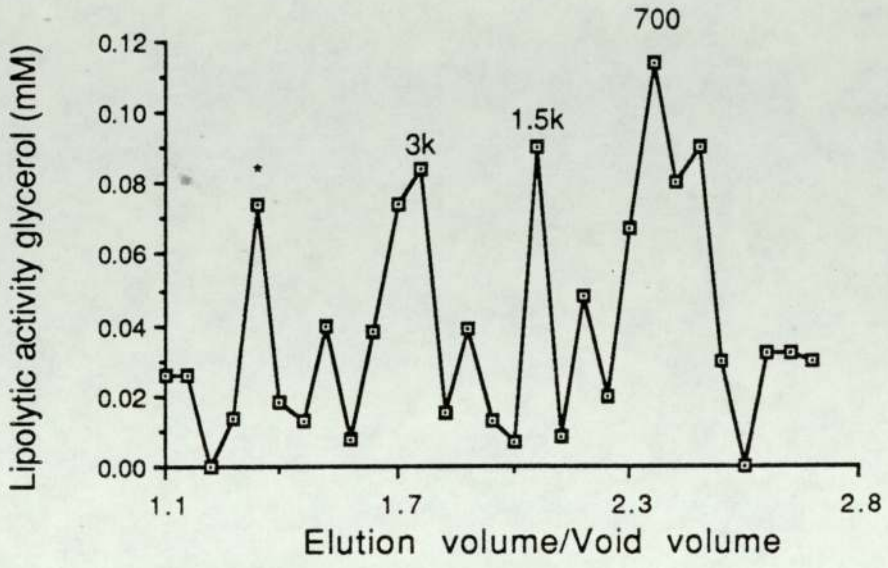




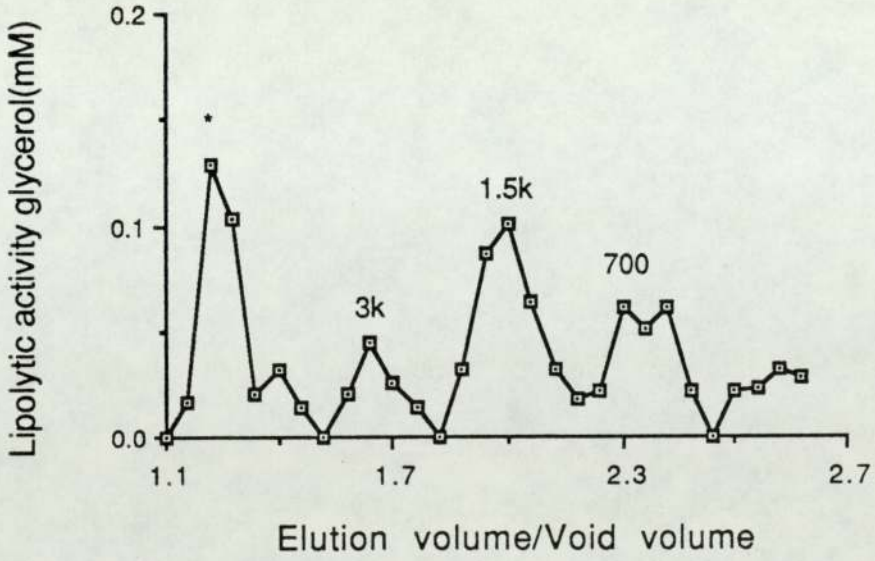
Lipolytic activity distribution pattern of 1.0ml fractions obtained from a Sephadex column eluted with 0.01M phosphate buffer pH8.0. Void volume = 16.0 ml.

Figure 62

SG50 chromatography of a sample(100ml) of urine from a cancer patient (R) with weight loss.



SG50 chromatography of a sample(100ml) of urine from a cancer patient (P) without weight loss.



Lipolytic activity distribution pattern of 1.0ml fractions obtained from sephadex G50 column eluted with 0.01M phosphate buffer pH8.0. Void volume= 17.0 ml.

less in patients without weight loss.

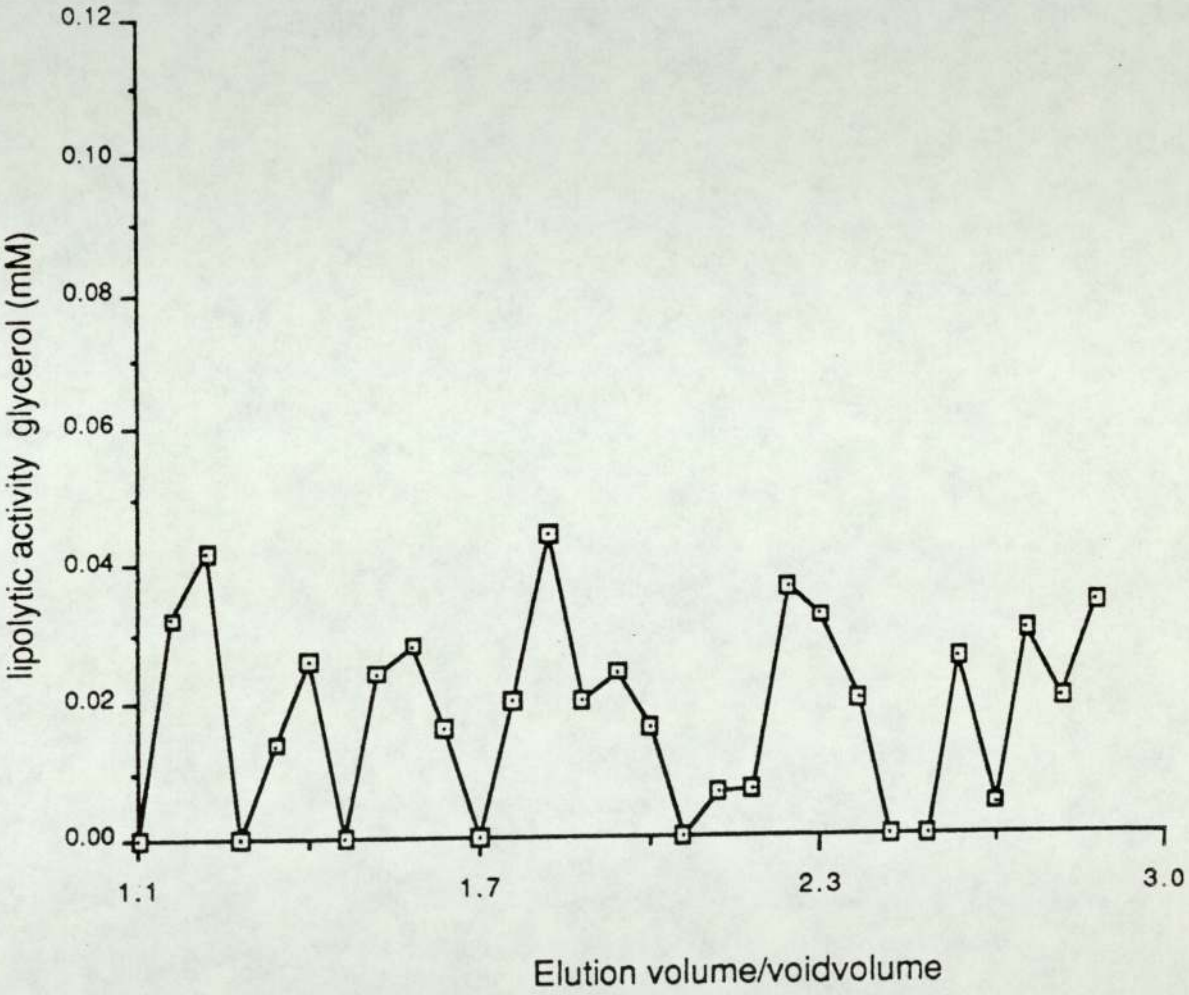
Lipolytic factors have been reported in the urine of non tumour bearing healthy individuals after fasting (Weil and Stetten 1947, Chalmers et al 1958, 1960). Thus the urine of six non tumour-bearing individuals was collected (128ml) after 24 h of starvation, concentrated and passed down a Sephadex G50 column. No peaks of activity were found in the molecular weight range 3 to 0.7k daltons (fig 63).

Human colon tumours were obtained from the operating theatre of the Queen Elizabeth Hospital, Birmingham and were examined for the presence of tumour lipolytic factors. 1.0ml of the colon tumour homogenate (R.E) that gave the highest lipolytic activity in vitro was subjected to Sephadex G50 column chromatography. Many lipolytic factors were found (including the 3 present in the MAC16 adenocarcinoma) of apparent molecular weight of 20, 5, 3, 1.5 and 0.7k daltons (fig 64).

All of the peaks obtained were slightly altered in their elution position due to variation in packing of the column and ageing of the column throughout the time it was being used. The 3k dalton peak ranged from 1.6-1.9 elution volume/void volume giving a molecular weight range of 3162 - 5623 daltons. This may also be due to the active components binding to other small molecules so varying the molecular weight determined. This is possible as it is consistent with the high adhesive properties of the material noticed during purification. Smaller molecular weight peaks did not vary as much, the 1.5k dalton peak having a molecular weight range of 1496 - 1259 daltons and the 0.7k dalton peak a

Figure 63

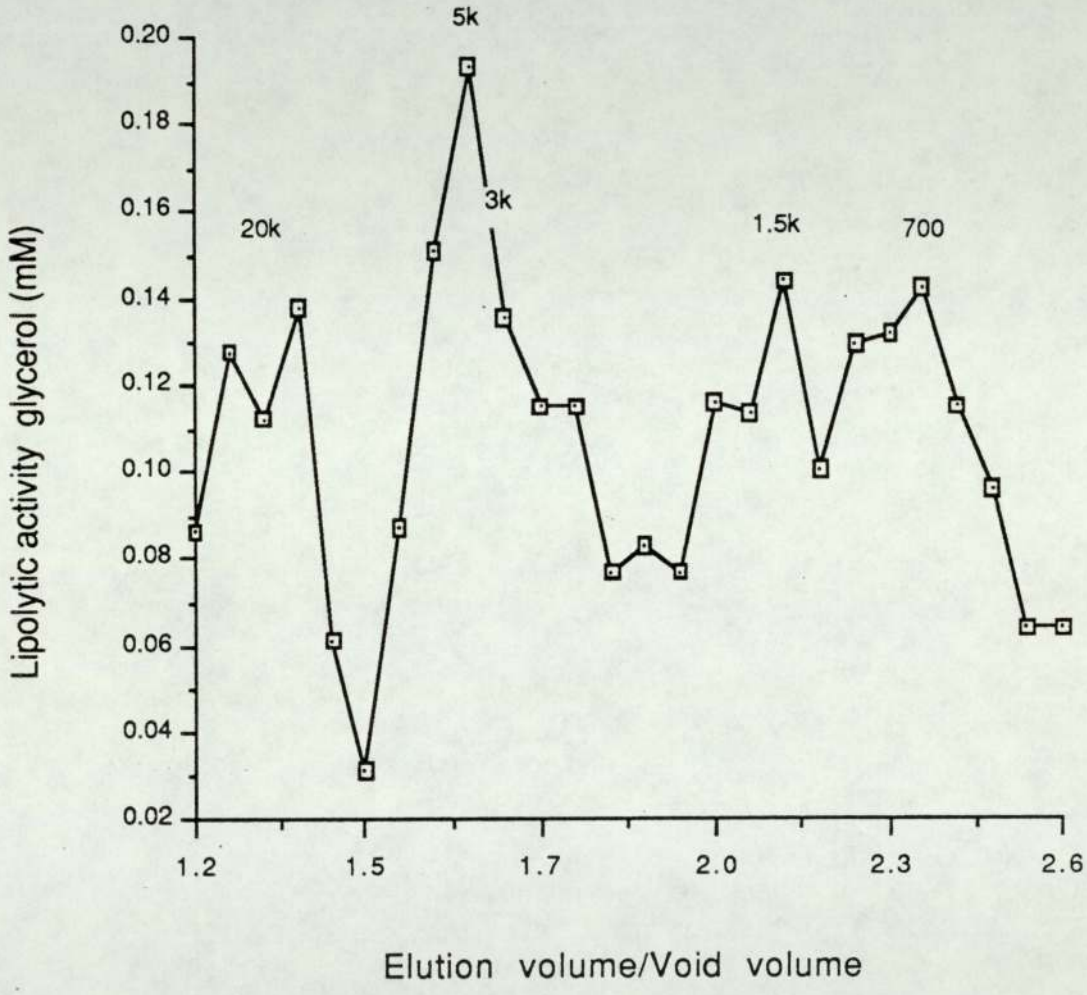
SG50 chromatography of a sample(128ml) of urine combined from control subjects after 24 hr starvation.



Lipolytic activity distribution pattern of 1.0ml fractions obtained a sephadex G50 column eluted with 0.01M phosphate buffer pH8.0. Void volume= 17.0 ml.

Figure 64

SG50 chromatography of a sample(1.0ml) of a human(R.E) colon tumour homogenate



Lipolytic activity distribution pattern of 1.0ml fractions obtained from a G50 sephadex column eluted with 0.01M phosphate buffer pH8.0.Void volume= 17.0 ml.

molecular weight range of 1000 - 794 daltons.

4.5.3 Discussion

In the MAC16 adenocarcinoma we have identified 3 lipolytic factors of apparent molecular weights of 3, 1.5, and 0.7k daltons. The molecular weights of these factors can not be determined accurately until the precise structures are known. To show our cachectic model is analogous to the human situation we must be able to show the presence of these factors in cachectic cancer patients. These factors are circulatory and so should be detectable in the serum and because of their low molecular weight possibly also in the urine of such patients. Non-tumour bearing healthy individuals were examined as controls and showed no evidence of any lipolytic factors of the same molecular weight as seen in the MAC16 tumour in their urine or serum even after starvation. In the serum of cancer patients however, lipolytic factors were found of the same apparent molecular weights of 3, 1.5 and 0.7k daltons. In patients with Alzheimers disease the only lipolytic factor found was of a molecular weight > 20k daltons. This larger lipolytic factor may be due to an enhanced level of a normal lipolytic hormone or lipase present in Alzheimers syndrome. Further work is needed to determine the origin of this lipolytic factor. The nutritional status in Alzheimers patients is difficult to determine. Maclennan (1975) studied patients in a long stay geriatric hospital including a group with dementia and showed after seven days the demented patients had a less adequate nutritional intake compared to the

patients without any mental disability and patients with a physical disability. Thus even when patients are hospitalised malnutrition can occur due to several factors, 1. they may have difficulty in recognizing and communicating their need for food, 2. the brain damage that exists may cause a suppression of appetite and feeding problems and 3. limited cooperation at feeding times by the patient (Maclennan et al 1975).

In burns patients many larger lipolytic factors were enhanced but there were no peaks of molecular weight < 3.0k daltons. There was, however, an overall increase in background lipolytic activity in this area. This high lipolytic activity seen in a patient with a burn injury is most likely due to high levels of catecholamines in the plasma. High levels of catecholamines in plasma and rate of catecholamine excretion have been reported in burned patients (Mason ,1979 and Newson, 1973). The weight loss experienced in these patients is probably due to a failure to provide a supranormal caloric intake needed during the hypermetabolic phase (Curreri, 1978).

Lipolytic factors of the same apparent molecular weight as in cancer patients were also found in the urine of mice bearing the MAC16 tumour. This showed a factor of higher molecular weight (>20k daltons) plus the three factors previously identified. On further treatment of the factor of high molecular weight with 0.5M NaCl it appeared to dissociate into the three factors of low molecular weight (3 to 0.7k daltons). It is possible that this high molecular weight factor is an aggregate of the low molecular weight

material or due to non specific adsorption of the low molecular weight material to proteins in the urine.The question remains as to whether these factors are products made exclusively by the tumour or whether they are produced by a unique action of the tumour to break down a normal/natural lipolytic hormone or lipase.

Mice bearing the MAC13 tumour exhibited only 2 of the lipolytic factors of apparent molecular weight 1.5 and 0.7k daltons . This relates to the tumour which also expressed only the lipolytic factors of apparent molecular weight of 1.5k and 700 dalton .

It appears that the presence of these factors in the urine is dependent on a tumour being present and not the amount of weight loss experienced as seen in the serum. In cancer patients with no weight loss all 3 lipolytic factors were still identified plus a lipolytic factor of high molecular weight as seen in the urine of the MAC16 tumour bearing mice. The problem with correlation of weight loss and the amount of factor present is that the bioassay used may not be quantitative. In the bioassay a set number of adipocytes(10^5) were incubated with potential lipolytic factors each time and this puts a limit on the maximum production of glycerol possible. In addition the assay alone without chromatography does not distinguish between the factors we are interested in and normal lipolytic hormones or lipases. Therefore the assay gives more qualitative results than quantitative but further modifications could result in a quantitative assay. A clinical problem was the recording of weight loss as it is

dependent on the patients recollection of how much weight they have lost during a certain period of time. In addition with patients who have operable or treatable cancers eg. cervical cancer, they are obviously treated quickly before any major weight loss can manifest itself.

We have studied the lipolytic activities in operable human colon tumour samples. The colon tumour exhibited many lipolytic factors, a larger peak at apparent molecular weight of 20k daltons plus an unidentified peak at 5k daltons and in addition the 3 lipolytic factors displayed in the MAC16 adenocarcinoma with apparent molecular weights of 3k, 1.5k and 700 daltons. This again illustrates that these factors are tumour associated. This may prove useful for the diagnosis of cancer and if important in tumour growth or maintenance of structure could be specific targets of attack in the treatment of cachexia or tumour growth.

After purification and identification of these lipolytic factors, the next question that arises is how do these factors work? In the next section the mechanism of action of the catabolic factors produced by the MAC16 adenocarcinoma is discussed.

4.6 Mechanism of action of the catabolic factors produced by the MAC16 adenocarcinoma.

4.6.1 Introduction

Hormones and growth factors, which are too large to penetrate the cell, exert their effects using second messenger molecules within the cell. Occupancy of the cell surface receptors initiates the production of active messenger molecules such as adenosine 3', 5'-monophosphate (cyclic AMP). The cyclic AMP binds to the regulatory subunits of a protein kinase. Binding of cyclic AMP to the protein kinase releases the free catalytic subunit from the dissociated protein kinase. This catalytic unit phosphorylates specific cellular proteins. Cyclic AMP has a role in proliferation and diferentiation of cells and has subsequently been investigated for its role in tumour growth.

4.6.1.1 Role of cyclic AMP in lipolysis

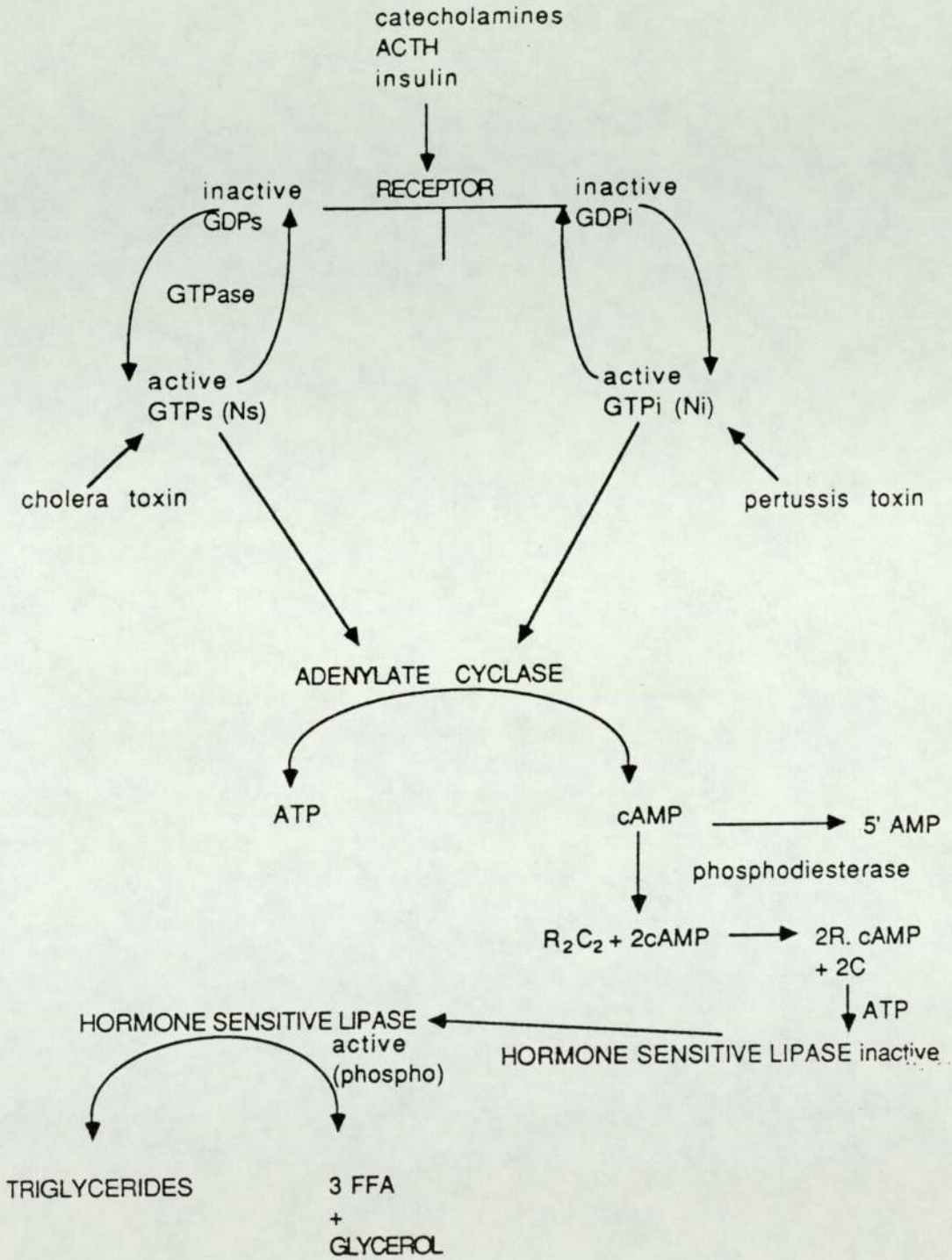
In 1956 cyclic AMP was identified as a key regulatory agent in most mammalian tissues. Considerable evidence has been reported on the cyclic AMP-mediated lipolytic action of many hormones. Adenosine has been reported to play an endogenous modulatory role in fat cell lipolysis (Galon et al 1988). It appears to be the factor that determines the basal lipolytic rate as well as being involved in response to stimulatory and inhibitory agents. The presence of a catecholamine-sensitive adenyl cyclase system was first shown in cell free preparations of adipose tissue

(Sutherland and Rall , 1960). Steroid hormones such as ACTH and glucagon also stimulated an increase in cyclic AMP levels in fat pads in vitro (Butcher et al 1966) and acted synergistically with methylxanthines on lipolysis and cyclic AMP levels (Butcher and Sutherland 1967). These lipolytic hormones appear to increase intracellular cyclic AMP levels by activating the adenylate cyclase system rather than by inhibiting cyclic AMP phosphodiesterases. Oestrogens in various mammalian species regulate body weight and adiposity. They have also been shown to increase adenylate cyclase activity in rat fat cells . This would explain their in vivo lipolytic properties (Pasquier et al 1988).

The in vitro lipolytic effect of epinephrine was antagonised by insulin. Sutherland (1966) further reported a decrease in cyclic AMP levels after treatment with insulin. Adenylate cyclase stimulation and inhibition by hormones is mediated by two seperate guanine nucleotide binding regulatory components Ns and Ni respectively (Lambert et al 1985). Cholera toxin activates the Ns guanine nucleotide binding protein. After a lag time of 30 minutes cholera toxin inhibits GTPase by ADP-ribosylation thus increasing the active form of the enzyme ,thus it stimulates cyclic AMP accumulation and lipolysis linearly for up to two hours. Bordetella pertussis toxin has a different mechanism of action, it inactivates the Ni guanine nucleotide binding protein. It presents a linear increase of GTP, cyclic AMP accumulation and lipolysis for up to two hours. These toxins have a direct effect on the adenylate cyclase system in contrast to hormone action which are

Figure 65

The role of cyclic AMP in lipolysis



receptor mediated (see fig 65). It has been identified that the adrenaline and isoprenaline stimulation of cyclic AMP is mediated via a B-adrenergic receptor. Occupancy of this receptor causes a release of cyclic AMP into the cytosol. Cyclic AMP activates a protein kinase which in turn mediates the phosphorylation of the regulatory site of the hormone sensitive lipase (Lonnroth and Smith 1986). Addition of insulin causes a rapid decrease in the phosphorylation of the lipase possibly by activation of a phosphodiesterase. Researchers have recently concluded that hormones such as adrenaline, ACTH or glucagon stimulate the Ns guanine protein where as insulin stimulates the Ni guanine protein. These proteins are regulated by a GTPase enzyme that hydrolyses the active GTP protein to the inactive GDP protein (see fig 65). Fat breakdown is important in the regulation of body weight . Thus the adenylate cyclase system may play a major role in cachexia.

4.6.1.3 Role of cyclic AMP in proteolysis

Within recent years it has become clear that cyclic AMP is capable of stimulating the synthesis or induction of a number of enzymes. Cyclic AMP is known to inhibit the overall rate of amino acid incorporation into protein (Pryor and Berthet 1960). Depressed protein synthesis is an important feature of muscle wasting experienced in cachexia (Rennie et al 1983). Whether this is regulated by the adenylate cyclase system remains to be determined.

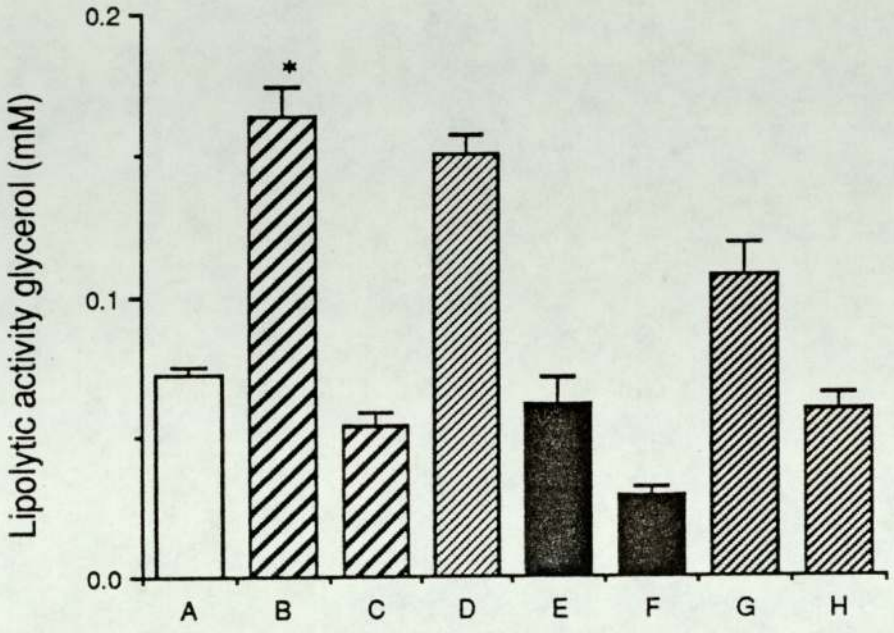
4.6.2 Results

4.6.2.1 Mechanism of action of lipolytic factors produced by the MAC16 adenocarcinoma

Isobutylmethylxanthine an inhibitor of cyclic AMP phosphodiesterases at a concentration of 0.1mM significantly enhanced the glycerol release from murine adipocytes caused by the partially purified lipolytic factor (fig 66). However at a concentration of 1.0mM isobutylmethylxanthine did not have any effect on the lipolytic action of the tumour factor. In contrast the lipolytic effect of noradrenaline was not affected by isobutylmethylxanthine at either concentration. These results may be due to the limit of lipolysis that can occur with 10^5 adipocytes. A further stimulation of lipolysis by isobutylmethylxanthine may have produced a competitive inhibitory effect on the noradrenaline induced lipolytic activity. The action of the partially purified factor on cyclic AMP production in adipocytes was compared with the lipolytic activitiy induced by either ACTH or the β adrenergic agonist salbutamol . After a 10minute incubation the tumour factor , ACTH and salbutamol all increased cyclic AMP levels in the cell (fig 67a). However after a 1 h incubation the tumour factor and salbutamol maintained the enhanced cyclic AMP levels in the cell but the levels of cyclic AMP in the ACTH treated cells had fallen back to normal (fig 67b). Following a 2 h incubation the tumour factor still caused an increased cyclic AMP level in the cell. However ACTH and salbutamol treated cells had normal cyclic AMP levels (fig 67c).

Figure 66

Effect of Isobutylmethylxanthine on lipolytic factor

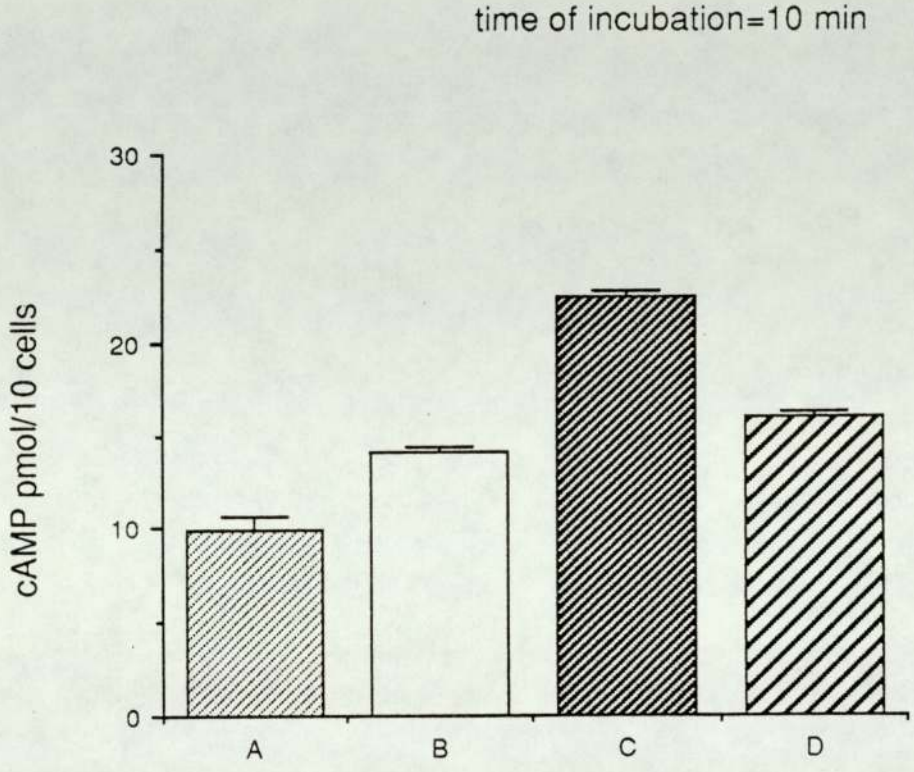


A. 100µl factor alone
B. Factor + 0.1mM isobutylmethylxanthine
C. Factor + 1.0mM Isobutylmethylxanthine
D. 20µg/ml Noradrenaline
E. 0.1mM Isobutylmethylxanthine
F. 1.0mM Isobutylmethylxanthine
G. Noradrenaline(20µg/ml) + 0.1mM Isobutylmethylxanthine
H. Noradrenaline(20µg/ml) + 1.0mM Isobutylmethylxanthine

Results are expressed as the mean±S.E.M. The number of experiments performed were 3 to 6. The basal lipolytic activity has been subtracted from the values above. * significant difference = $p<0.001$ compared to factor alone.

Figure 67 (a)

Effect of factor, ACTH and salbutamol on cAMP levels

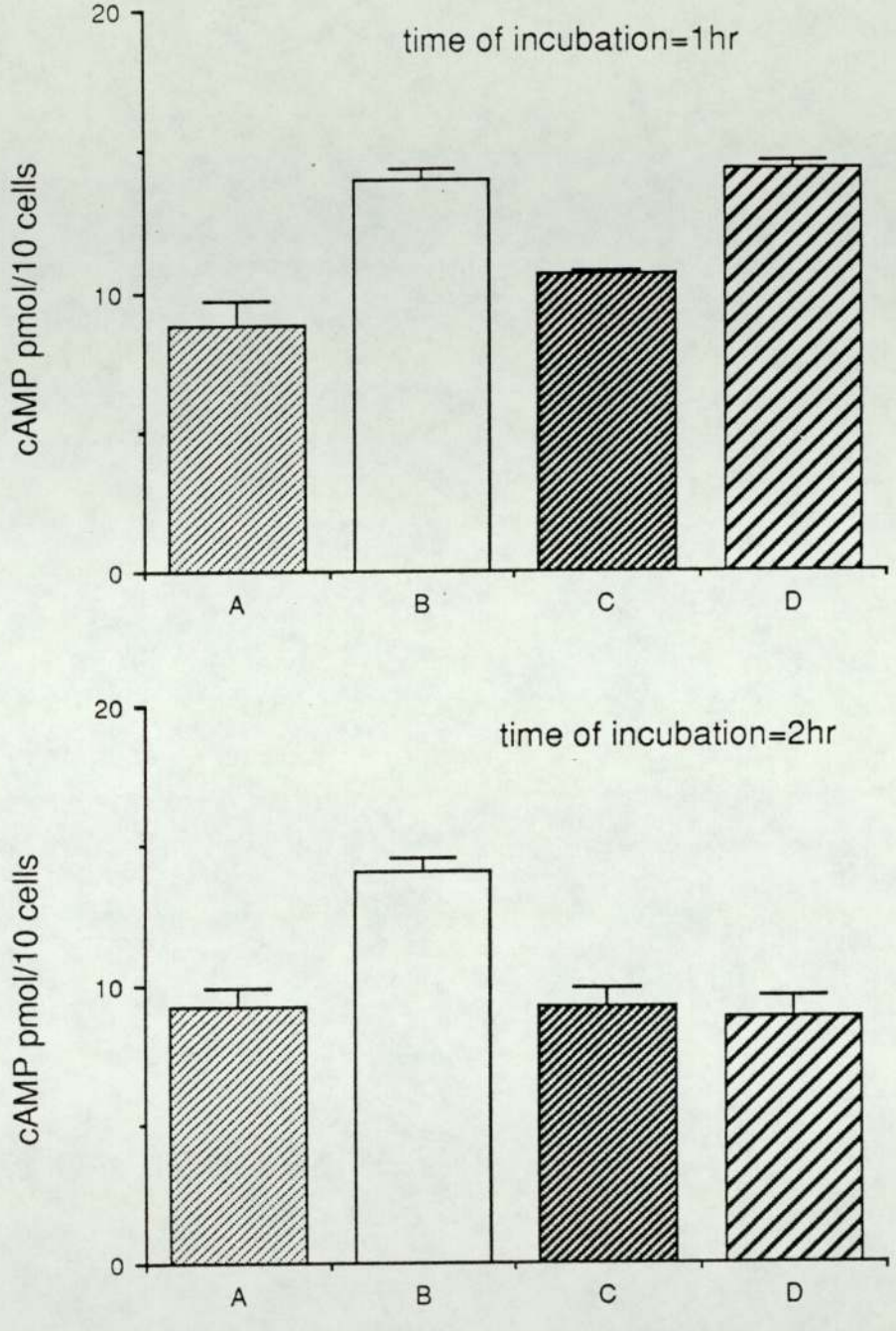


A. Control cells only
B. 50µl factor
C. 25 units ACTH
D. 0.165mM salbutamol

Results are expressed as the mean± S.E.M. The number of experiments performed were 3 to 4.

Figure 67 (b) + (c)

Effect of factor, ACTH and salbutamol on cAMP levels



A. Control cells only
B. 50µl factor
C. 25 units ACTH
D. 0.165mM salbutamol

Results are expressed as the Mean ± S.E.M. The number of experiments performed were 3 to 4.

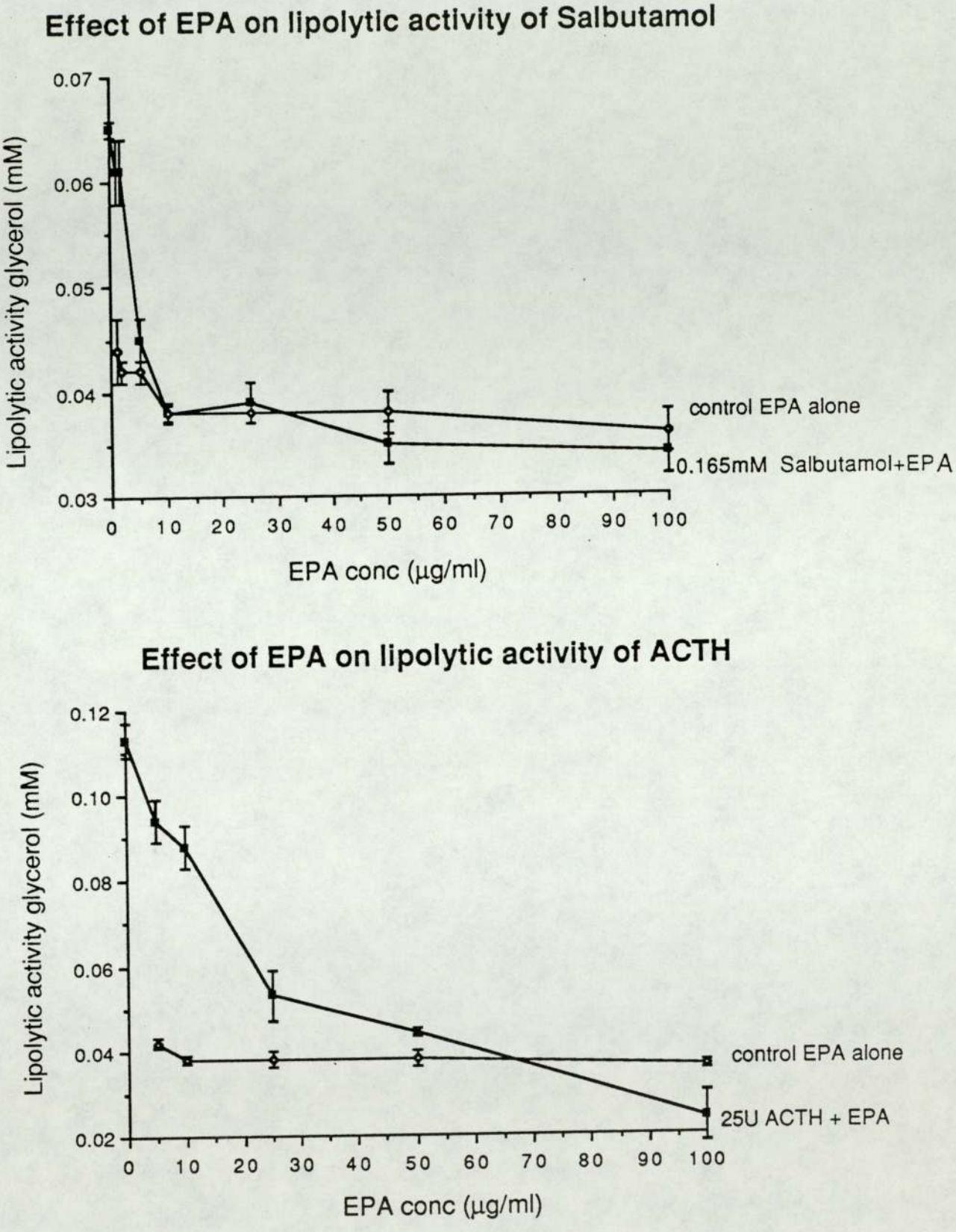
Combination of the factor and salbutamol showed their lipolytic activities were not synergistic. TNF (10^5units/ml) had no effect on cyclic AMP levels in the adipocytes. Ecoisapentaenoic acid (EPA) an *in vitro* inhibitor of the factor (see section 4.7) also inhibited the lipolytic activities of ACTH and salbutamol (fig 68). The inhibitory action of EPA on the lipolytic activitiy induced by the factor , ACTH or salbutamol was correlated with a reduction in the stimulated levels of cyclic AMP in the cell (fig 69).

4.6.2.2 Mechanism of action of proteolytic factors produced by the MAC16 adenocarcinoma

After a ten minute incubation the MAC16 tumour proteolytic factor increased cyclic AMP levels in mouse diaphragm (fig 70). No effect on cyclic AMP levels was observed after incubation for 1 or 2 h. EPA as shown earlier inhibits the proteolytic activity of the factor produced by the MAC16 tumour (see section 4.7). The inhibitory action of EPA on the proteolytic activity induced by the tumour effect was correlated with a reduction in the stimulated levels of cyclic AMP in mouse diaphragms (fig 70). Prostaglandin E_2 series also act by increasing cyclic AMP levels in mouse diaphragm (fig 71). However TNF (10^5 units/ml) did not increase cyclic AMP levels in the mouse diaphragm (fig 71).

The lipolytic factor produced by the MAC16 tumour showed some evidence of proteolytic activity when incubated for 2 h at 37°C with mouse diaphragm (fig 72). This

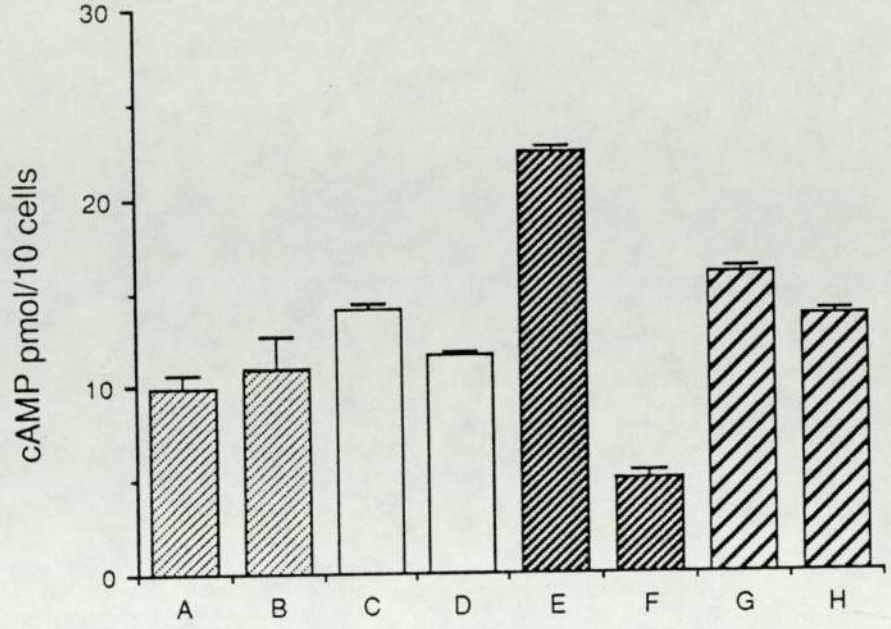
Figure 68



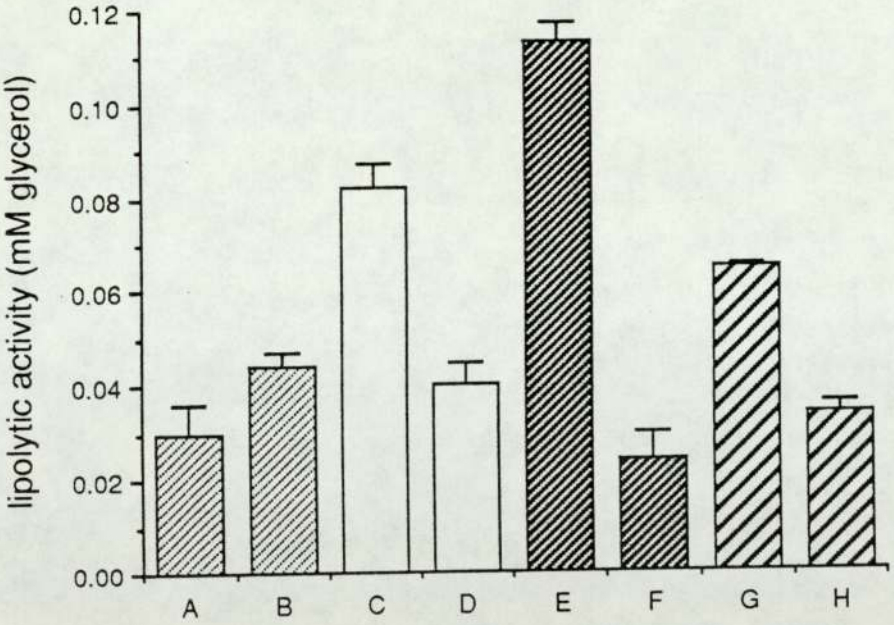
Results are expressed as the Mean ± S.E.M of 3 to 5 experiments.

Figure 69

Effect of EPA on factor,ACTH and salbutamol cAMP levels



Effect of EPA on lipolytic activity of factor,ACTH and salbutamol.

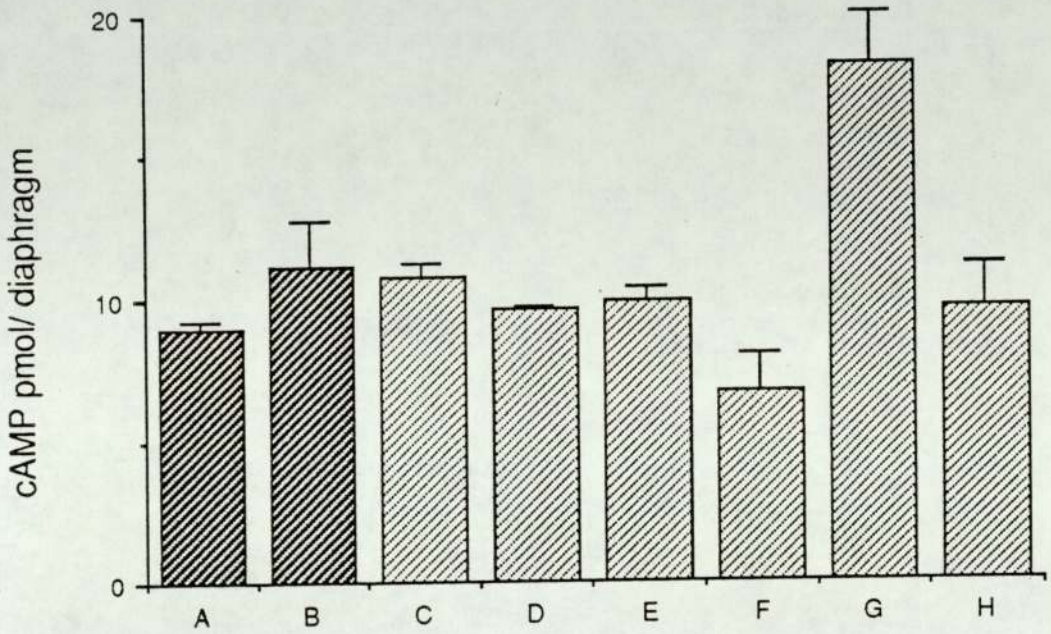


A. Control cells only
B. 0.331mM EPA
C. 50µl factor
D. 50µl factor + 0.331mM EPA
E. 25units ACTH
F. 25 units ACTH + 0.331mM EPA
G. 0.165mM Salbutamol
H. 0.165mM Salbutamol + 0.331mM EPA

Results are expressed as the Mean ± S.E.M of 3 to 4 experiments.

Figure 70

Effect of proteolytic factor and EPA on cAMP levels

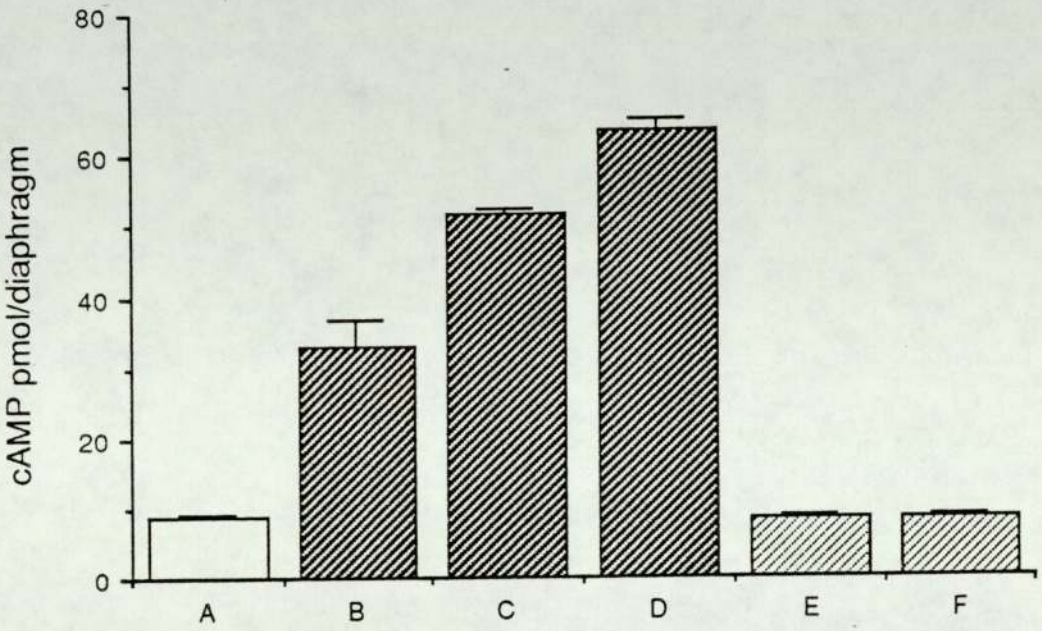


A. control level in mouse diaphragm
B. 0.331mM EPA
C. 50µl proteolytic factor
D. 50µl factor + 0.331mMEPA
E. 100µl proteolytic factor
F. 100µl factor + 0.331mM EPA
G. 150µl proteolytic factor
H. 150µl factor + 0.331mM EPA

The results are expressed as the Mean ± S.E.M of 3 to 4 experiments.

Figure 71

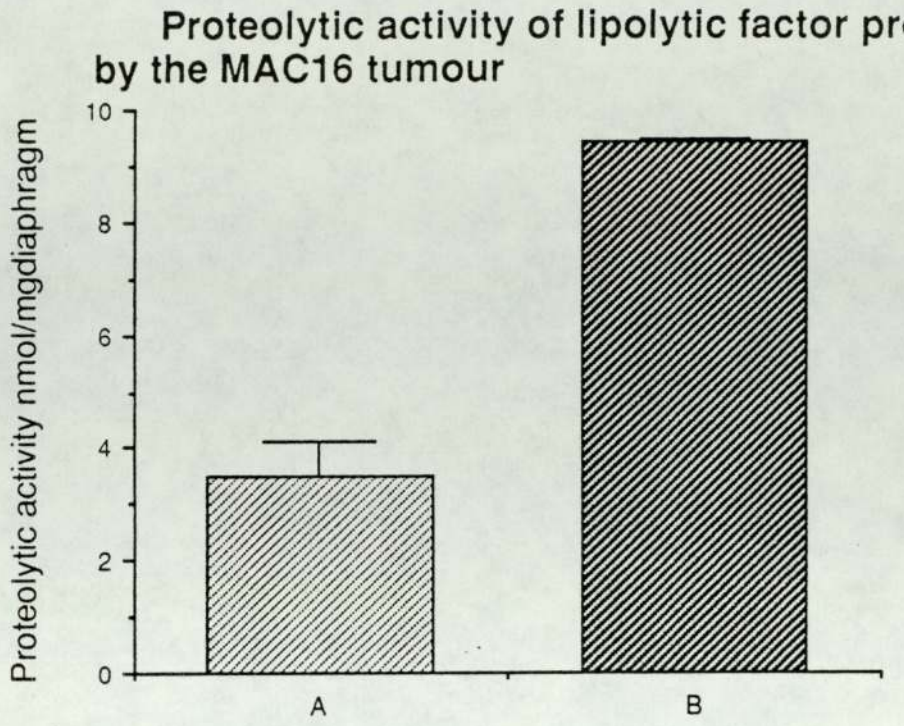
Effect of prostaglandins and TNF on cAMP levels



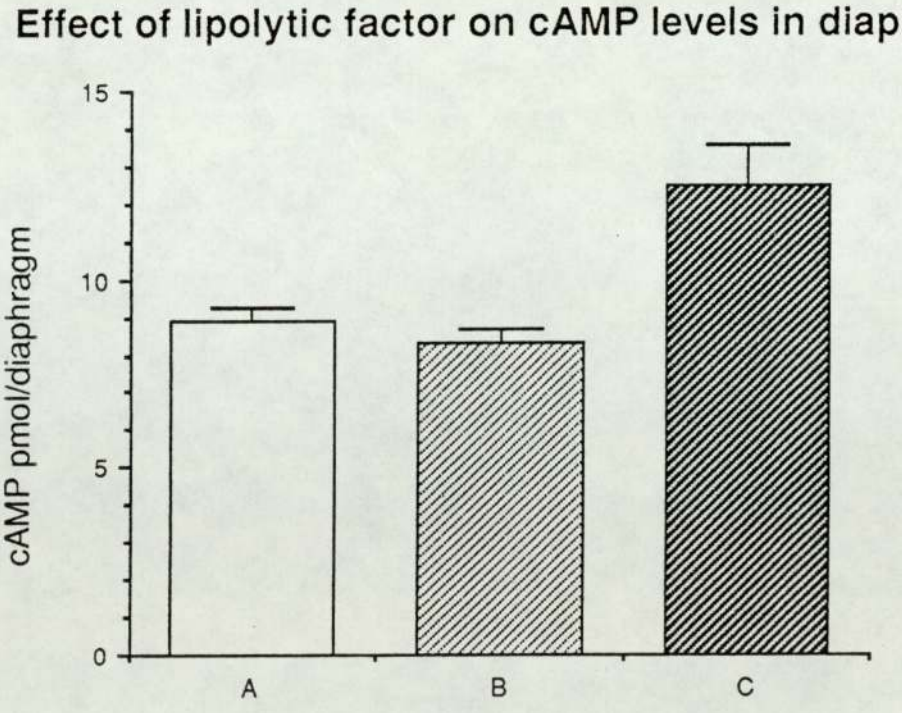
A. control
B. 5µg/ml PGE
C. 10µg/ml PGE
D. 20µg/ml PGE
E. 10^5U/ml TNF
F. 10^5U/ml TNF + 1.0mg/ml Indomethacin

The results are expressed as the Mean ± S.E.M of 3 to 4 experiments.

Figure 72



A. 50µl lipolytic factor
B. 100µl lipolytic factor



A. control
B. 50µl lipolytic factor
C. 100µl lipolytic factor

The results are expressed as the Mean± S.E.M of 3 to 4 experiments.

correlated with its ability to increase cyclic AMP levels in mouse diaphragms (fig 72).

4.6.3 Discussion

Methylxanthines are known to stimulate cyclic AMP formation and lipolysis. They inhibit cyclic AMP breakdown by reducing phosphodiesterase activity and antagonising the inhibitory effects of adenosine. Adenosine stimulates the guanine inhibitory binding protein. This effect is blocked by isobutylmethylxanthine (Londos et al 1978). Thus the actions of the lipolytic factors isolated from the MAC16 adenocarcinoma appeared to be mediated through the adenylate cyclase system as its effects were enhanced by isobutylmethylxanthine.

ACTH and salbutamol increased cyclic AMP in the cell, via different cell surface receptors. Their enhancement of cyclic AMP levels was only transient and after 2 h cyclic AMP levels in the cell returned to normal. In contrast to this the tumour factor still increased cyclic AMP levels after 2 h. This was similar to the cyclic AMP stimulation caused by cholera and pertussis toxin. Cholera and pertussis toxin do not possess a receptor mediated action. Instead they bind directly to the guanine binding proteins of the adenylate cyclase system (Lambert et al 1985). Thus the action of the tumour lipolytic factors may be due either to a direct effect on the adenylate cyclase system or a prolonged stimulation of a cell surface receptor. Salbutamol and the factor do not act via the same receptor as no competitive effect was observed between them . Thus

the factor does not cause lipolysis via the stimulation of the β-adrenergic receptor. EPA inhibited the lipolytic activities of the factor, ACTH and salbutamol and its effects were directly related to the increase or decrease in cyclic AMP produced. Therefore it too could be acting via a direct effect on the adenylate cyclase system, perhaps by stimulating the inhibitory GTP protein.

The enhancement of cyclic AMP by the proteolytic tumour factor was only transient and after 1 h cyclic AMP levels in the diaphragms had returned to normal. This may indicate a receptor mediated action. EPA inhibited the proteolytic activity of the tumour proteolytic factor and its effects were directly related to the decrease in cyclic AMP produced. As stated before EPA may directly effect the adenylate cyclase system. As discussed in section 4.2 the proteolytic factors actions may be due to induction of prostaglandin E_2 release. This hypothesis is further substantiated as prostaglandin E_2 increased cyclic AMP levels similarly to the proteolytic factor produced by the MAC16 tumour. Many of the effects of TNF have been reported to be due to the production of prostaglandin E_2 (Dayer 1985). However its proteolytic effect (shown in section 4.2) was not due to increased cyclic AMP levels in mouse diaphragm, so does not seem to involve prostaglandin E_2.

The lipolytic factor produced by the MAC16 tumour also possessed some proteolytic activity, again this activity was mediated via the adenylate cyclase system.

Further work is needed to determine the exact site where the lipolytic factors, proteolytic factors and EPA act

in the adenylate cyclase system and in addition if there is a cell surface receptor specific for the lipolytic or proteolytic factor, to isolate it and to do further receptor binding studies.

Reversal of the action of these factors could lead to a treatment of cachexia in cancer patients. In the next section inhibitors of the catabolic factors produced by the MAC16 adenocarcinoma and their role in the reversal of cancer cachexia is discussed.

4.7 Inhibitors of the catabolic factors produced by the MAC16 adenocarcinoma and their role in the reversal of cancer cachexia.

4.7.1 Introduction

Cachexia is responsible for the severe morbidity and mortality seen in cancer patients and for a decreased tolerance to cancer treatment (Dewys et al 1980, Rivlin et al 1983, Heber et al 1986). In general attempts at alimentation or hyperalimentation have had some success in cases where the patient had a physical barrier to food ingestion (Tchekmedyian et al 1986). An example of this would be patients with head and neck cancer in whom chewing and swallowing was difficult. In other cases where this barrier did not occur there has been no evidence that alimentation by either enteral or parenteral routes have had any effect on the catabolic state or weight status of the patient. It has been shown that even with extra nutritional support in many patients with advanced cancer, loss of weight continues whilst they receive the extra calories which would be expected to result in weight gain (Heber et al 1986). A major problem with nutritional support is that it may cause nutritional stimulation of tumour growth (Nixon et al 1981a). Thus other methods of treatment are needed to treat cachexia in the cancer patient. We have examined three different groups of substances:

a) Normal metabolic controls eg. Insulin.

b) Dietary effects 1. changes in fat content of the diet. High fat diets containing medium chain triglycerides

(MCT). 2. changes in the carbohydrate content of the diet. Fish oil incorporated diets.

c) Postulated anticachectic drugs for example Megestrol acetate.

Insulin has anabolic effects that would oppose the catabolic effects of the tumour. It has been shown that administration of exogenous insulin in normal animals results in hyperphagia, weight gain and deposition of body fat (Moley et al 1985, Morrison 1982). Other effects of insulin reported are inhibition of breakdown of fat and protein and stimulation of fat and protein synthesis. Hence a use for insulin has been suggested in the treatment of cancer cachexia. Insulin treatment has been shown to stimulate food intake and increase host weight in three different rat strains with different tumour types (Moley et al 1983, Morrison 1982). It has been reported that treatment of cachectic and anorectic animals with insulin preserves fat stores and prevents depletion of protein without stimulating tumour growth (Moley et al 1985). In all of these situations a decreased food intake was a symptom of the experimental cachectic model used. Insulin has also been shown to inhibit some of the toxic effects produced by cachectin/TNF (Fraker et al 1987). This may be due to its reversal of the anorectic effect produced by cachectin/TNF.

Dietary changes are favourable as they are not toxic to the host. Ketone bodies (acetoacetate and 3-hydroxybutyrate) can be used as a source of energy for the brain in hypoglycaemia (Owen et al 1967). They also

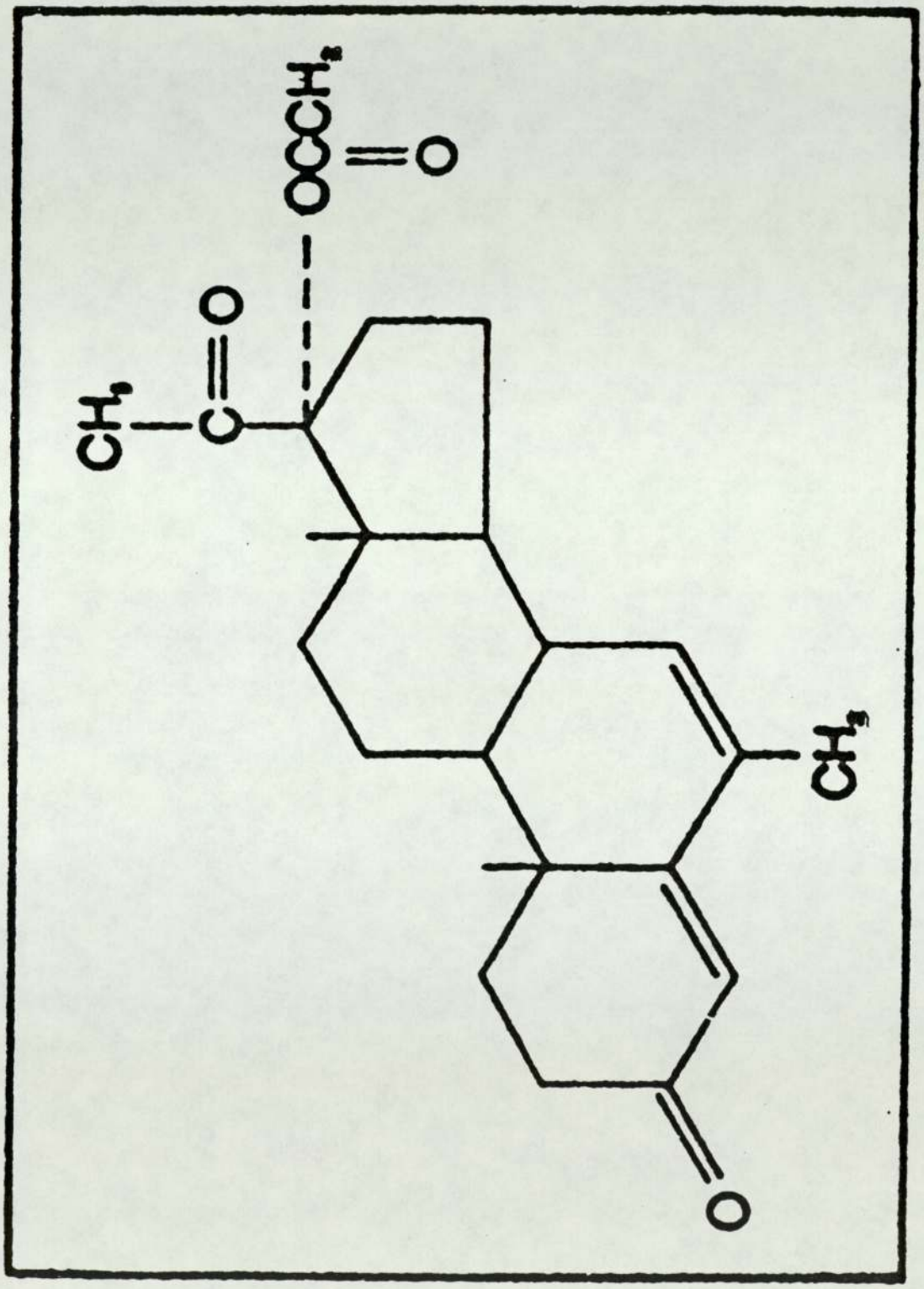
directly reduce protein degradation in muscle and stimulate insulin release from the pancreas (Hawkins et al 1971). Ketonuria is an uncommon phenomenon in cancer patients (Conyers et al 1979) and tumour bearing rodents (Bibby et al 1987). Ketonemia can be demonstrated in cancer patients and tumour bearing rodents provided with an exogenous supply of fatty acids. Hence there is no malfunction of the livers ability to synthesise ketone bodies (Magee et al 1979). Thus a low carbohydrate diet may reverse the cachexia seen in cancer patients. In addition it may reduce the rate of growth of tumours which depend on glucose as an energy source (Tisdale 1982, Tisdale et al 1987).

An alternative dietary change is to substitute the carbohydrate content of the diet by calories derived from fish oil. Fish oil is rich inω-3 type polyunsaturated fatty acids eicosapentaenoic acid (20:5 ω-3) (EPA) and docosahexaenoic acid (22:6ω-3) (DCH). These are essential fatty acids that are not made by the body but are a necessary requirement for growth. Interest in fish oils started in 1976 when it was shown that the incidence of cancer in Alaskan and Greenland Eskimos was low compared to Western Society (Waterhouse et al 1976). It was found that the diets of these Eskimos contained large amounts of oils derived from fish and seals (Bang, 1976). Dietary fish oil has been reported to reduce the size and number of preneoplastic lesions of the rat pancreas induced by L-azaserine (Oconnor et al 1985). It also reduces mammary tumour incidence and prolongs the tumour latency period in rats after administration of the carcinogen

N-methyl-N-nitrosourea (Jukowski et al 1985). A significant reduction in weight and size of the rat R3230 A.C mammary tumour (Karmali et al 1984) and a transplantable human prostate tumour DU-145 (Karmali et al 1987) was also observed after feeding a fish oil diet for 4 weeks. The growth of the BN472 a mammary adenocarcinoma was inhibited in rats which recieved a ω-3 fatty acid diet but not in those which recieved a ω-6 fatty acid diet (Kort et al 1987).Therefore it is not an increase in fat alone that causes these effects but specifically an increase in ω-3 fatty acids. Hence fish oil may reverse cachexia in cancer patients and in addition display an antitumour effect.

Recently many drug companies have become interested in the anticachectic properties of their drugs. At the moment Bristol Myers are investigating the anticachectic properties of their breast cancer drug Megestrol acetate (MEGACE). Megestrolacetate(17-acetoxy-6-methylpregna-4,6-diene-3,20-dione structural formula see fig 73) is an orally active synthetic derivative of the naturally occuring steroid hormone progestrone. It is licenced for use in the United Kingdom for the treatment of certain hormone dependent neoplasms, such as breast cancer and endometrial cancer. Megace also shows an antineoplastic effect in prostatic cancer (Johnson et al 1975). The antioestrogenic effect is believed to be responsible for the antitumour activity seen in breast cancer (Gregory et al 1985) and endometrial cancer (Wait 1973). Megestrol acetate has been observed anecdotally to produce weight gain in cancer patients. A phase I/II study using high dose megace for breast cancer revealed that

Figure 73



Megace

weight gain occured in 27% of the patients who recieved 160 mg per day. A marked weight gain was also noted in 27/28 patients given high doses of up to 1600 mg /day of megestrol acetate (Tchekmedyian et al 1986, 1987). A repeat of the above study showed that at doses of 1600 mg/day of megace, 80% of all patients exhibited weight gain (Aisner et al 1988). Hazard et al (1987) have shown an increase in glucose, high density lipids, cholesterol, triglycerides and phospholipids in the plasma of female rats given 5mg/kg/day of megace. In addition in vitro megestrol acetate has been shown to be a potent inducer of lipocyte differentiation (Harburger et al 1988). However the mechanism of action of megestrol acetate in inducing weight gain remains to be determined. A phase II study into the efficacy of high dose megestrol acetate in the treatment of cancer cachexia is presently taking place at the Queen Elizabeth Hospital, Birmingham.

4.7.2 Results

4.7.2.1 Inhibitors of the catabolic factors produced by the MAC16 adenocarcinoma in vitro

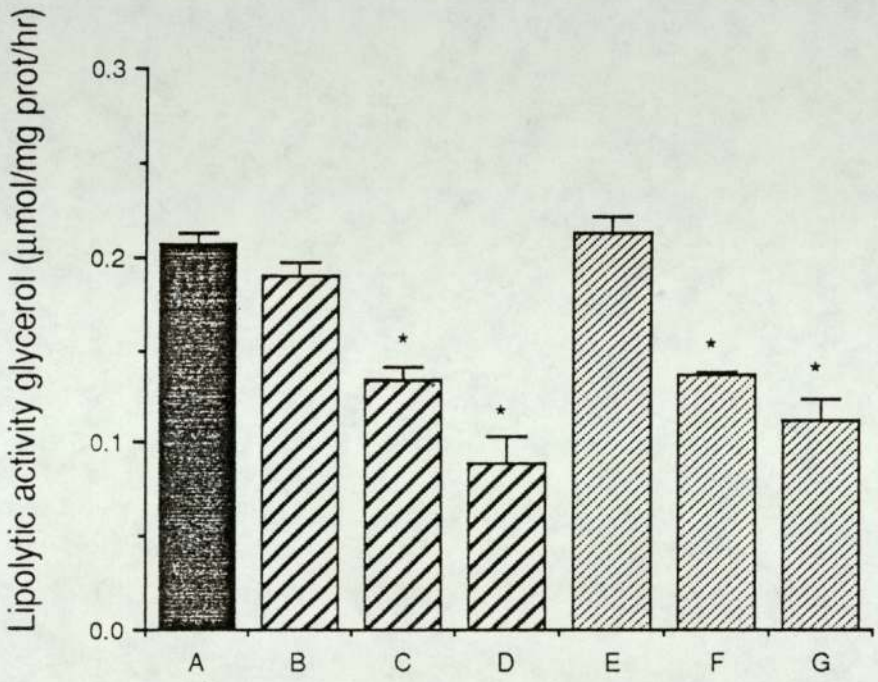
Insulin at doses of 10units/ml significantly inhibited the lipolytic and proteolytic activity of the MAC16 adenocarcinoma (fig 27 and 30). ketone bodies (3-hydroxybutyrate) similarly inhibited both lipolytic and proteolytic factors produced by the MAC16 tumour (fig 27 and 30). Sodium butyrate when compared with sodium 3-hydroxybutyrate had the same inhibitory effect over the

same concentration range (1.0 - 5.0mM) (fig 74). Fish oil is made up mainly of 2 polyunsaturated fatty acids, ecoisapentaenoic acid and docosahexaenoic acid. The effect of these and other fatty acids were examined in vitro. Docosahexaenoic acid and palmitic acid had no effect on the lipolytic or proteolytic activites of the MAC16 tumour. Ecoisapentanenoic acid(EPA) significantly inhibited both the lipolytic and proteolytic factors produced by the MAC16 adenocarcinoma (fig 75). EPA also inhibited the lipolytic activity expressed by the MAC13 adenocarcinoma (fig 76). Further investigations into the inhibition seen with EPA showed that this effect was reversed by prior addition of carnitine (fig 76). EPA exhibited effects on the glucose metabolism of the MAC13 cell line with an increased lactate production and decreased glucose utilisation. Again these effects were reversed by carnitine (fig 77).

Flavone acetic acid (FAA) an experimental antitumour agent is the only drug known to inhibit the MAC16 tumour growth in vivo. Hence its effect on the lipolytic factor produced by the MAC16 adenocarcinoma was examined. FAA showed a striking inhibitory effect on the MAC16 tumours lipolytic activity. However on further investigation this was shown to be an artifact due to FAA showing reduction in the NADH absorbance at 340nm (fig 78). This is a problem with many antitumour agents in that they interfere with the bioassay used to determine lipolytic activity. Megestrol acetate did not directly absorb at 340nm but did significantly inhibit the MAC16 tumours lipolytic activity at doses of 4 - 8mM (fig 79).

Figure 74

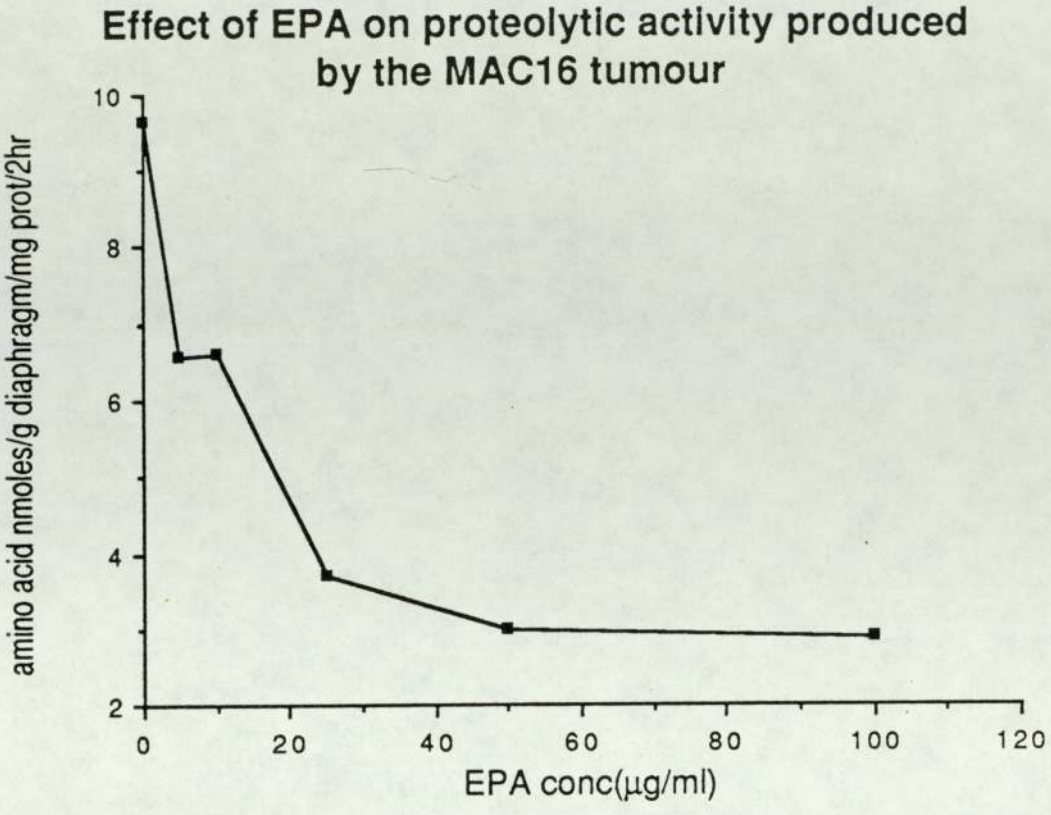
Comparison of sodium butyrate , sodium 3-hydroxybutyrate on inhibition of MAC16 tumour lipolytic activity

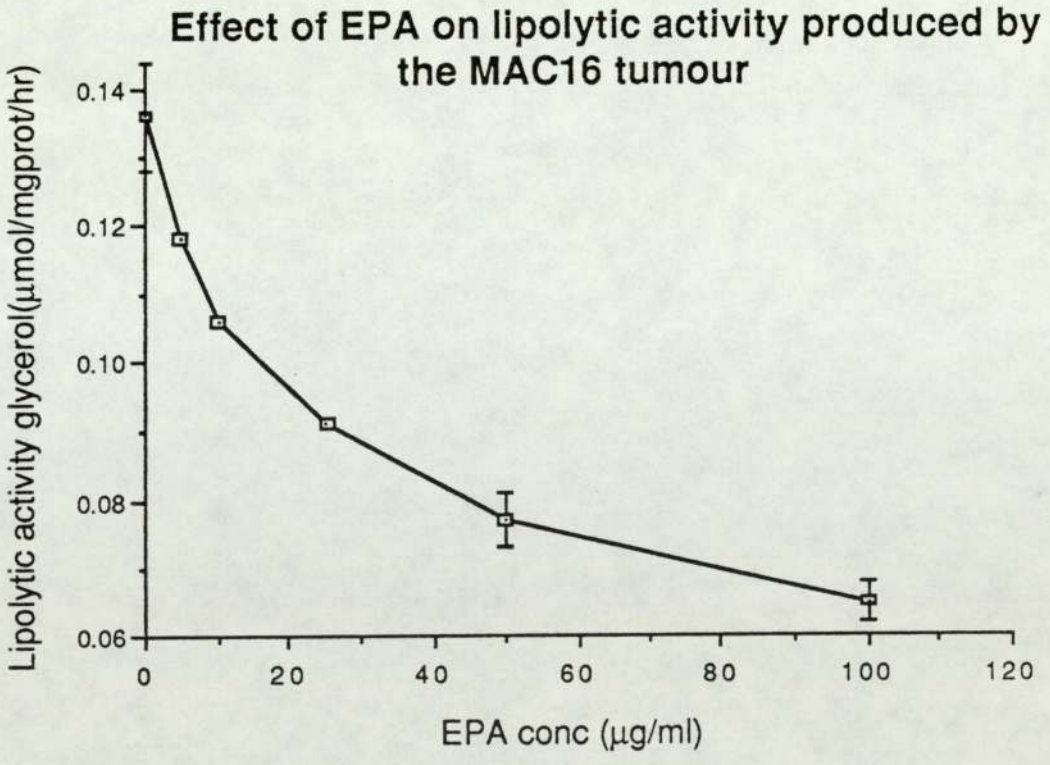


A. MAC16 tumour
B. MAC16 + 1.0mM Nabutyrate
C. MAC16 + 2.5mM Nabutyrate
D. MAC16 + 5.0mM Nabutyrate
E. MAC16 + 1.0mM Na3-hydroxybutyrate
F. MAC16 + 2.5mM Na3-hydroxybutyrate
G. MAC16 + 5.0mM Na3-hydroxybutyrate

The results are expressed as Mean ±S.E.M. The number of experiments performed were 3 to 5. Significant difference * $p<0.001$ compared to the MAC16 tumour.

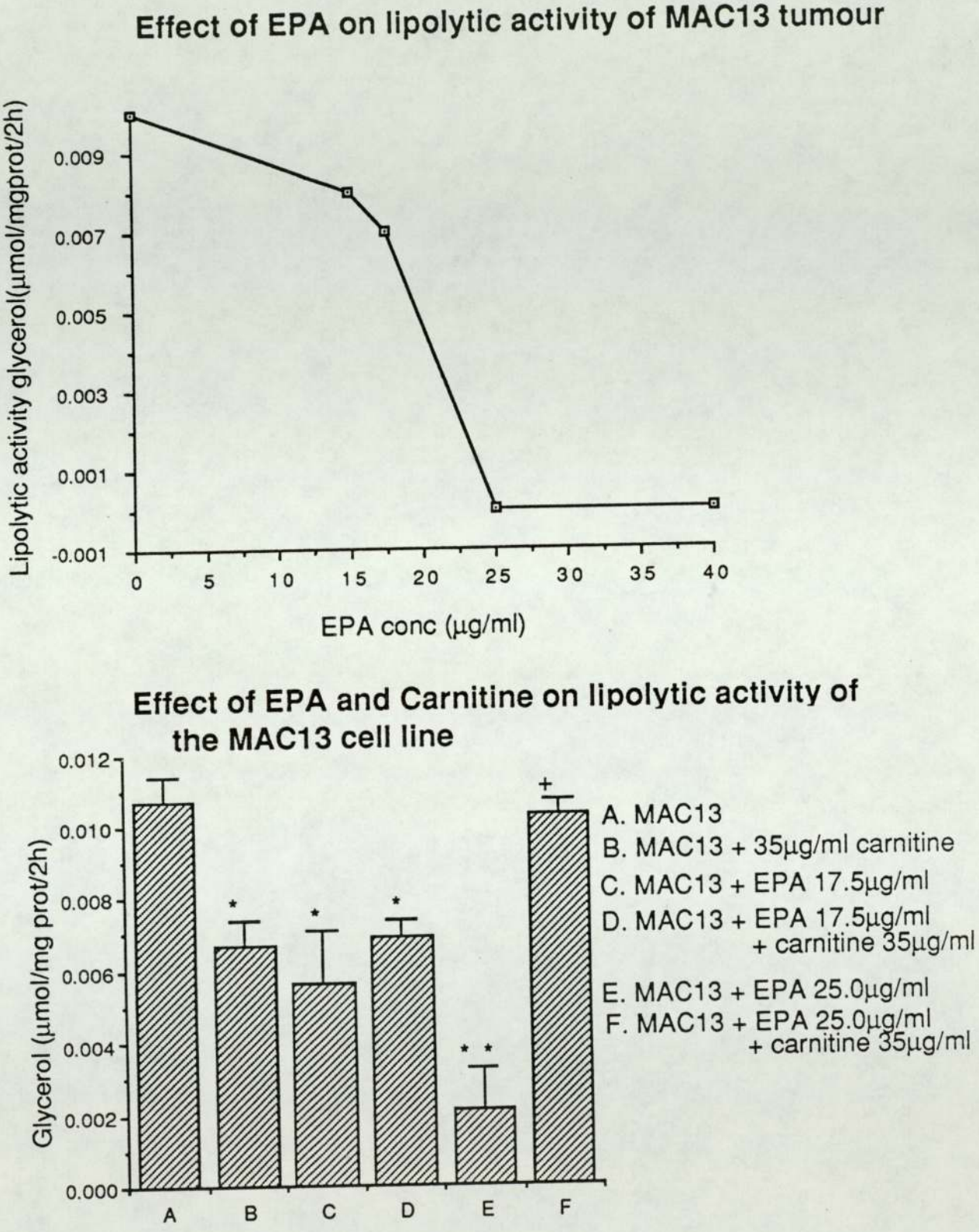
Figure 75





Results are expressed as mean where the number of experiments performed were 2 and the mean±S.E.M ,where the number of experiments performed was 3to4.

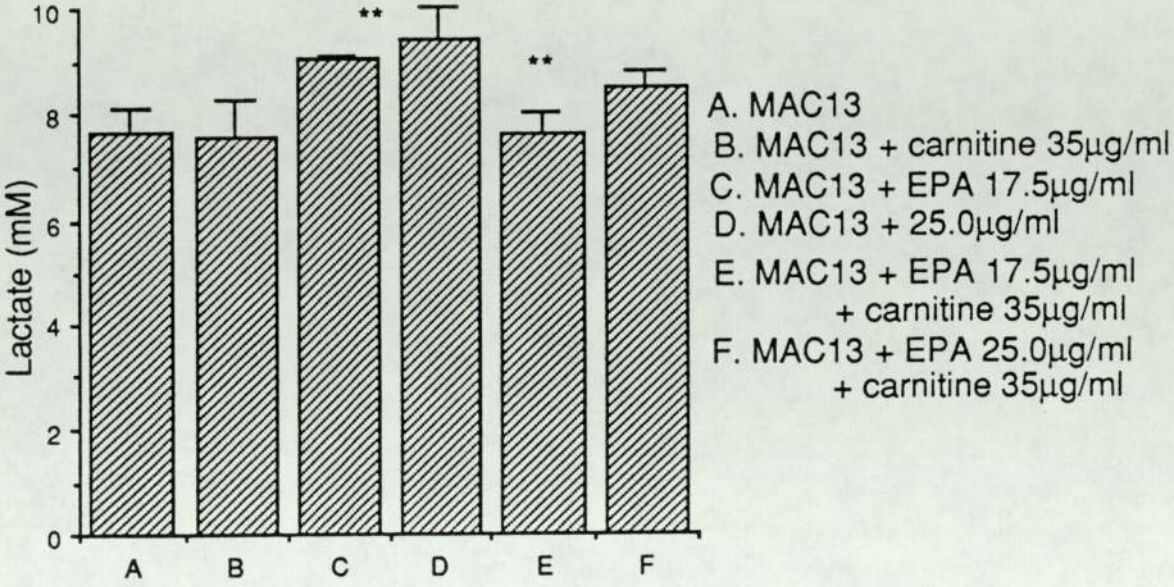
Figure 76



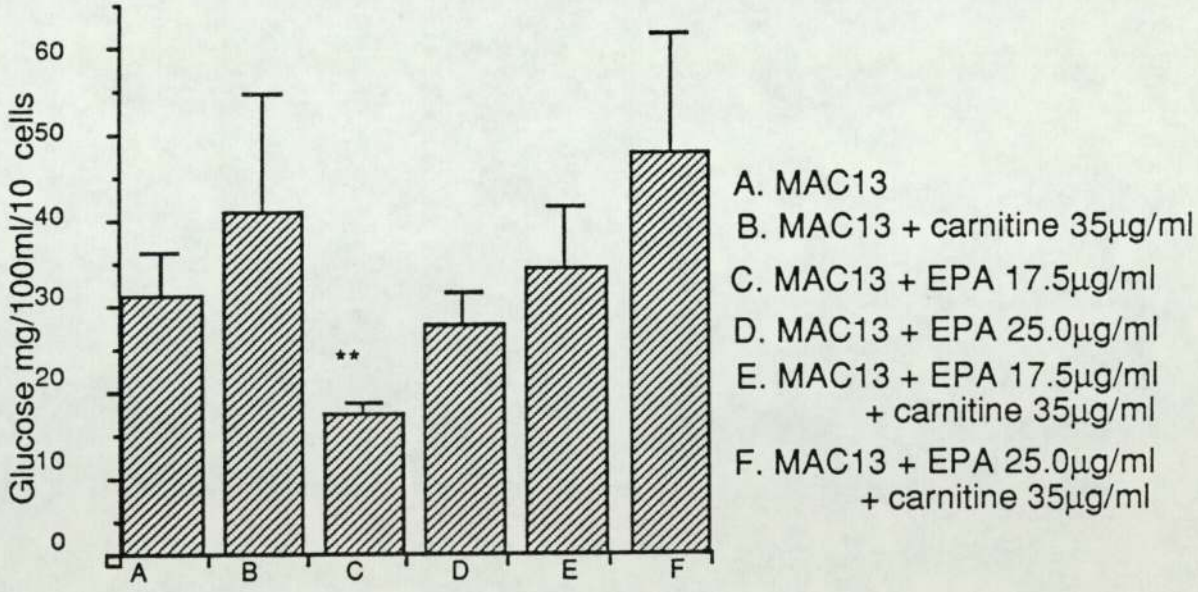
Results are expressed as mean ±S.E.M. The number of individual experiments performed were 2 to 9. Significant difference *p<0.01, **P<0.001 compared to MAC13 value. + p<0.001 compared to EPA 25.0µg/ml value.

Figure 77

Effect of EPA/Carnitine on lactate production by MAC13 cells

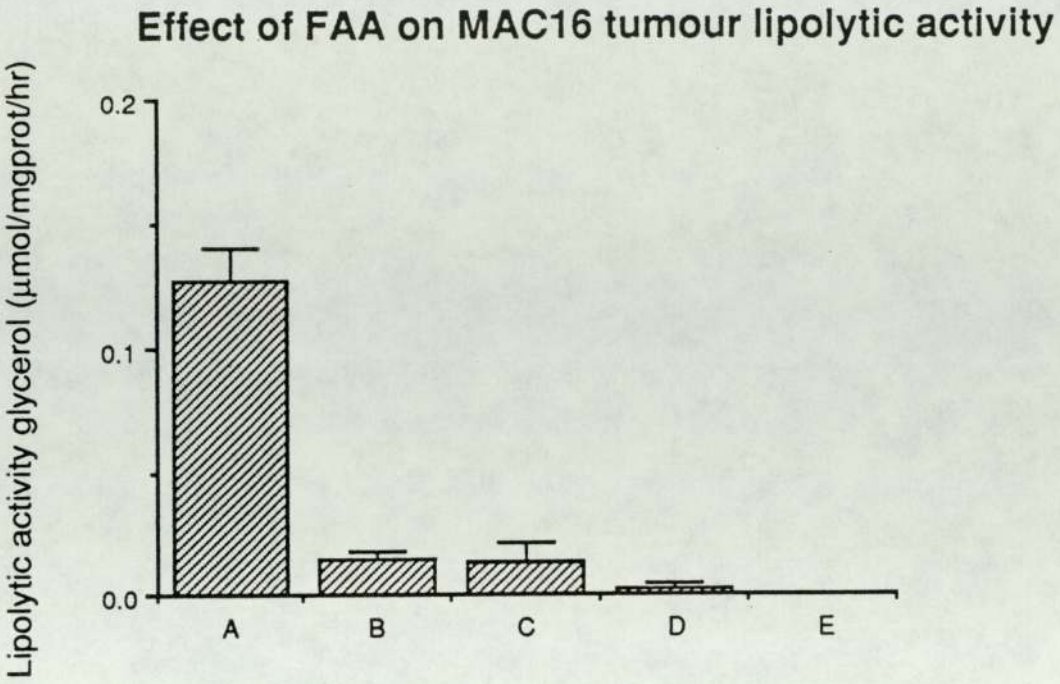


Effect of EPA/carnitine on glucose utilization by MAC13 cells

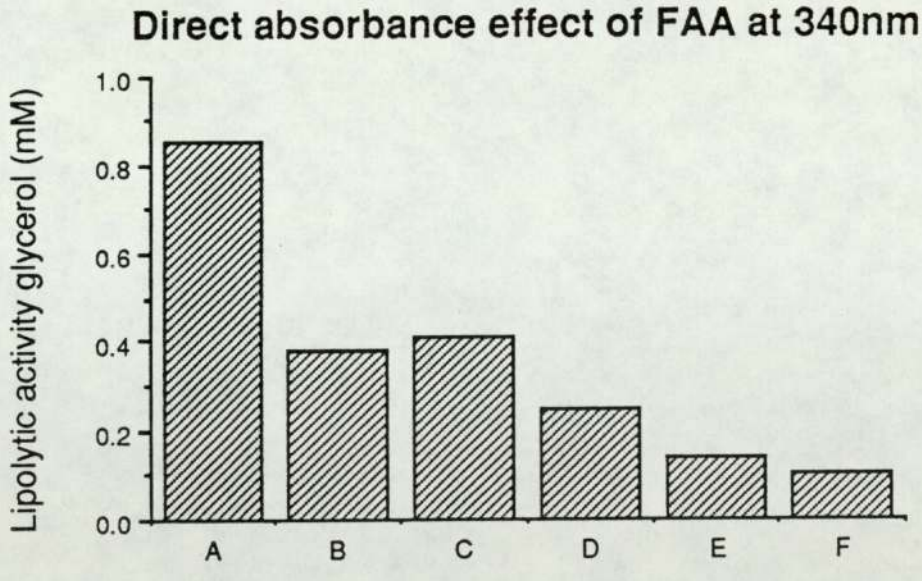


Results are expressed as mean± S.E.M. The number of individual experiments performed were 3to 6 . Significant difference ** $p<0.001$ compared to MAC13 value.

Figure 78



A. MAC16 tumour
B. MAC16 + FAA(10µg/ml)
C. MAC16 + FAA(25µg/ml)
D MAC16 + FAA(50µg/ml)
E.MAC16 + FAA(100µg/ml)

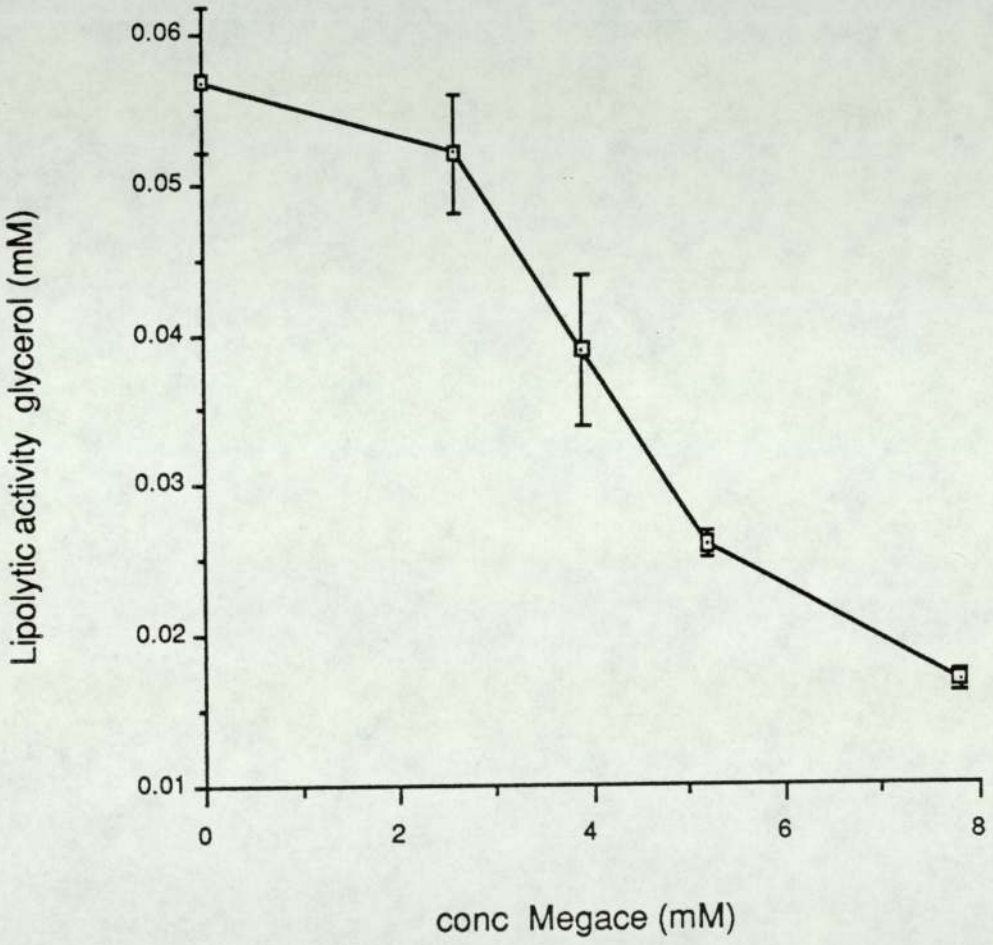


A. Glycerol 1.0mM
B. Glycerol 1.0mM + FAA(400µg/ml)
C. Glycerol 0.5mM
D. Glycerol 0.5mM + FAA(200µg/ml)
E. Glycerol0.1MM
F. Glycerol 0.1MM + FAA(20µg/ml)

Results are expressed as the mean where the number of experiments was 2 and Mean ±S.E.M where 3 to 5 experiments were performed.

Figure 79

In vitro Effect of MEGACE on MAC16 lipolytic activity



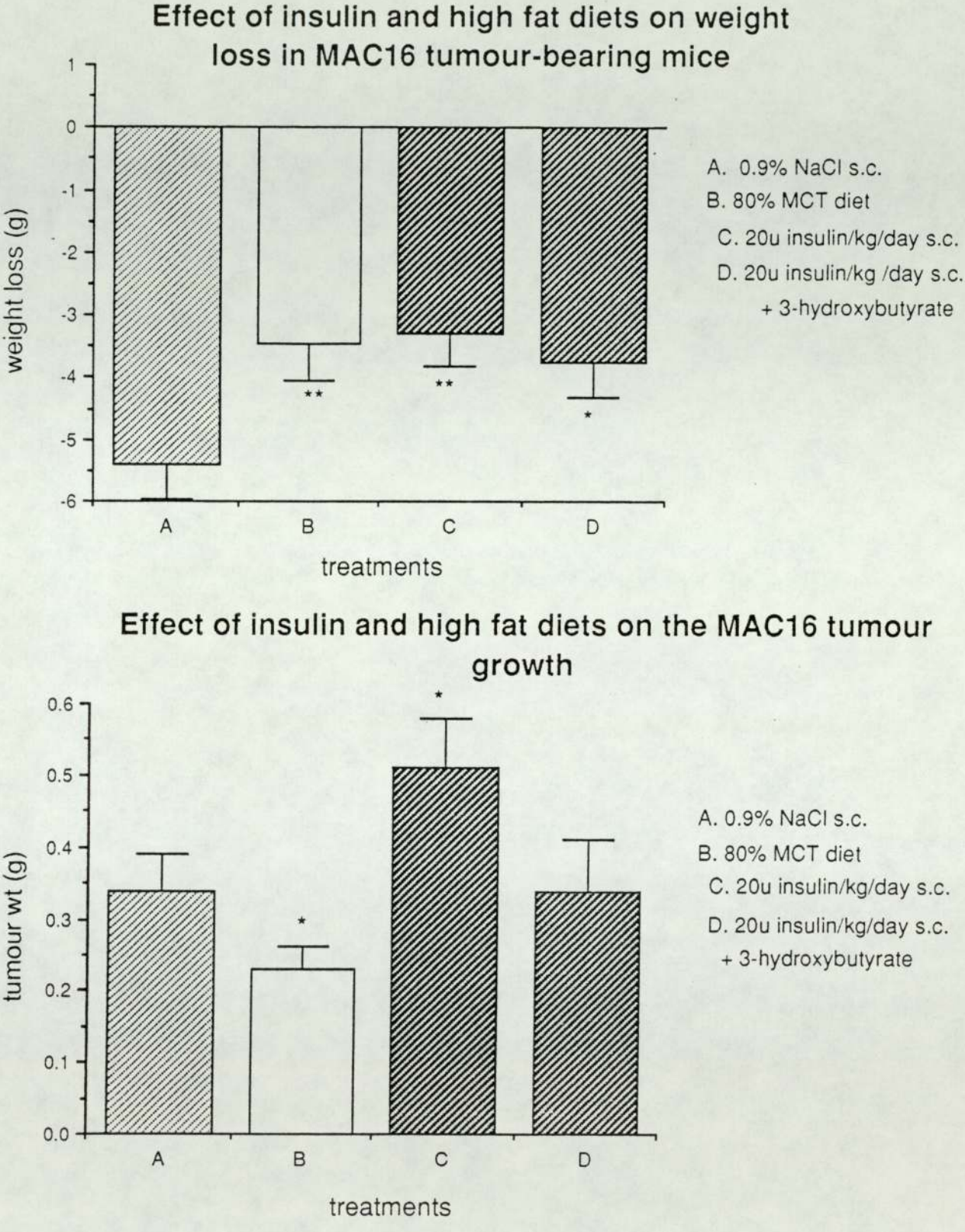
The results are expressed as the Mean± S.E.M.
The number of experiments performed were 3.

Thus insulin, 80% MCT diet, fish oil diet and megestrol acetate have been investigated in vivo.

4.7.2.2 Reversal of cachexia in vivo

Insulin significantly reduced the weight loss seen in the animals bearing the MAC16 tumour at a dose of 20 units/kg/day given for 8 days. This reduction in weight loss was not accompanied by any changes in the intake of food or water . However insulin apparently stimulated tumour growth as it significantly increased tumour size in these animals (fig 80). When the mice were fed an 80% MCT diet for 8 days a significant reduction in weight loss was observed. This was not accompanied by a change in food or water intake. The MCT diet showed a slight antitumour effect with a significant reduction in tumour size (fig 80). When insulin and 3-hydroxybutyrate were given together no synergistic effect was seen. The reduction in weight loss was associated with less fat breakdown and no muscle proteolysis (table 16). When MAC16 tumour-bearing mice were given 3-hydroxybutyrate alone there was no significant effect on the weight loss experienced compared to control MAC16 tumour-bearing animals. Nitrogen excretion in these animals was also reduced compared to the control MAC16 tumour bearing animals (table 17). Animals bearing the MAC16 tumour showed a reduced blood glucose level when compared with non tumour bearing control and in the insulin treated animals a further reduction in blood glucose occurred (table 18). In some cases this caused hypoglycaemic shock and death. In the MCT treated animals

Figure 80



The results are expressed as the Mean ±S.E.M . The number of animals per group was 5 to 10 . Significant difference *p< 0.05, **p<0.001.

Table 16

Effect of insulin and high fat diet (MCT) on body composition male NMRI mice bearing the MAC16 adenocarcinoma.

Tumour	Treatment	Carcass fat (g)	Muscle dry weight (thigh+gastrocnemius) (g)
None	0.9%NaCl	1.70±0.09**	0.090±0.003**
None	20U insulin/kg/day	1.64±0.10**	0.098±0.005*
MAC16	0.9%NaCl	0.58±0.11	0.070±0.002
MAC16	80%MCT diet	1.00±0.17*	0.080±0.003
MAC16	15U insulin/kg/day	1.07±0.11*	0.083±0.003*
MAC16	20U insulin/kg/day	1.00±0.10*	0.080±0.002
MAC16	20u insulin/kg/day +3-hydroxybutyrate	0.92±0.13	0.075±0.004
MAC16	3-hydroxybutyrate	0.65±0.12	0.070±0.003

The results are expressed as the Mean±S.E.M. The number of animals used were 6 to 12 per group. Significant difference *$p<0.05$, **$p<0.0004$ from MAC16 bearing animals injected with 0.9% NaCl.

Table 17

Effect of insulin and high fat diet on nitrogen excretion in NMRI mice bearing the MAC16 adenocarcinoma.

Tumour	treatment	total nitrogen input g/24h	total nitrogen output g/24h	Nitrogen balance g/24h
None	0.9%NaCl	0.048±0.007	0.030±0.003	0.018±0.007
MAC16	0.9%NaCl	0.044±0.005	0.042±0.003+	0.004±0.007+
MAC16	80%MCT diet	0.047±0.012	0.024±0.004*	0.023±0.007
MAC16	20U insulin/kg/day	0.039±0.006	0.024±0.007*	0.025±0.007+
MAC16	20u insulin/kg/day +3-hydroxybutyrate	0.039±0.013	0.024±0.010*	0.025±0.010+
MAC16	3-hydroxybutyrate	0.048±0.007	0.038±0.004	0.010±0.007

Tumour	Treatment	Urea g/24h	Ammonia mg/24h	Creatinin mg/24h
None	0.9%NaCl	0.028±0.03	0.391±0.050	0.644±0.05
MAC16	0.9%NaCl	0.041±0.003++	0.415±0.047	0.866±0.1
MAC16	80%MCT diet	0.023±0.004*	0.614±0.165	1.46±0.25
MAC16	20U insulin/kg/day	0.023±0.007*	0.787±0.357	0.484±0.2
MAC16	20U insullin/kg/day +3-hydroxybutyrate	0.023±0.009*	2.232±1.016**	0.409±0.1
MAC16	3-hydroxybutyrate	0.037±0.004++	6.48±1.01	0.742±0.1

The results are expressed as the Mean±S.E.M for 6 to 12 animals per group. Significant difference +$p<0.007$, ++$p<0.002$ compared with non tumour bearing and *$p<0.005$, **$p<0.0001$ compared with MAC16 tumour bearing animals injected 0.9% NaCl.

Table 18

Effect of insulin and high fat diet (MCT) on plasma metabolite levels in male NMRI mice bearing the MAC16 adenocarcinoma.

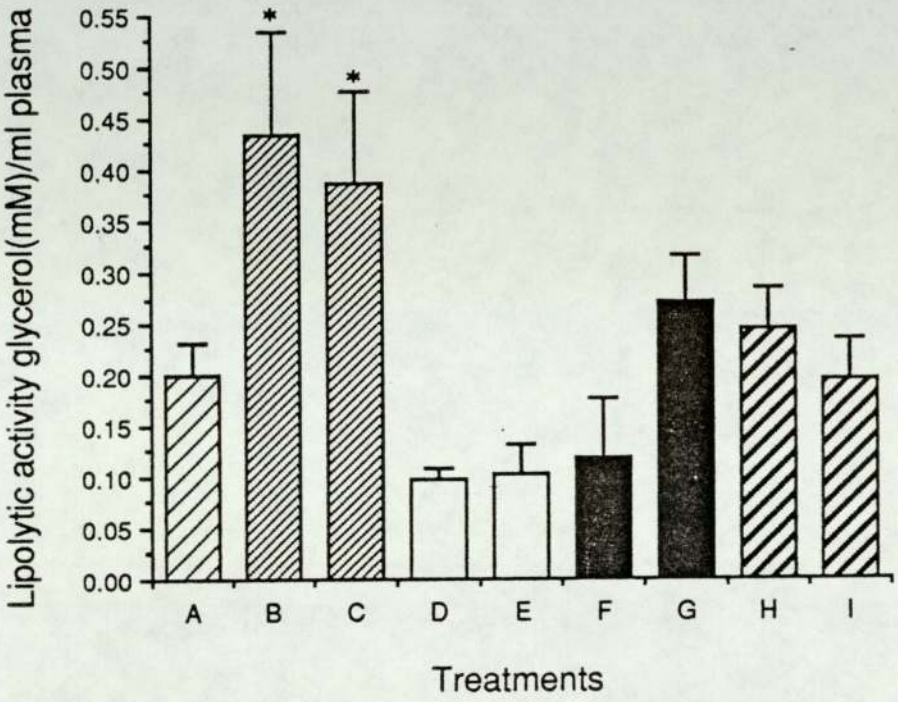
Tumour	Treatment	Glucose mM	FFA mM	Acetoacetate µM	3-Hydroxy butyrate µM
none	0.9%NaCl.	6.76±0.24	1.01±0.06	40±4	105±19
none	20ul/kg/d.	7.57±0.24	0.72±0.07	37±8	92±28
MAC16	0.9%NaCl	5.52±0.43*	0.36±0.02**	34±6	70±14
MAC16	80%MCT	5.60±0.36*	0.48±0.09**	74±7**	241±22**
MAC16	20ul/kg/d	4.22±0.76**	0.43±0.11**	39±8	81±19
MAC16	20ul/kg/d +3-hydroxy -butyrate	4.10±0.18**	0.49±0.06**	83±6**	265±30**

there was no significant change in the blood glucose level compared to the control MAC16 tumour-bearing animals. A high level of ketone bodies was present only in the plasma of the MCT treated mice (table 18). In both the insulin and MCT treated groups the levels of plasma FFA were increased compared to the control MAC16 tumour-bearing animals but were still significantly reduced compared to the non tumour-bearing animals. Fish oil was investigated *in vivo* and completely reversed any weight loss seen in MAC16 tumour bearing animals, accompanied by an antitumour effect (unpublished results, J. Dhesi). Insulin increased the amount of circulatory lipolytic factors in the plasma while in contrast fish oil decreased the amount of circulatory lipolytic factors (fig 81). However fish oil when in combination with MCT had no effect on the levels of circulatory lipolytic factors.

Preliminary experiments into the effects of megestrol acetate in cachexia have shown a slight reduction in the weight loss caused by the MAC16 tumour (fig 82). Mice bearing the MAC16 tumour were given 3.0mg/day Megace for 8 days. However, the effect was similar to insulin in that it caused an increase in tumour growth (fig 82). In addition megestrol acetate significantly increased food intake (fig 83). On increasing the dose of Megace to 3.0mg/mouse twice a day a significant reduction in the weight loss caused by the MAC16 tumour was seen (fig 84). The reduction in weight loss was again accompanied by an increase in tumour growth and an increased fluid and food intake (fig 84 and fig 85). The Megace had no significant effect on the plasma meta-

Figure 81

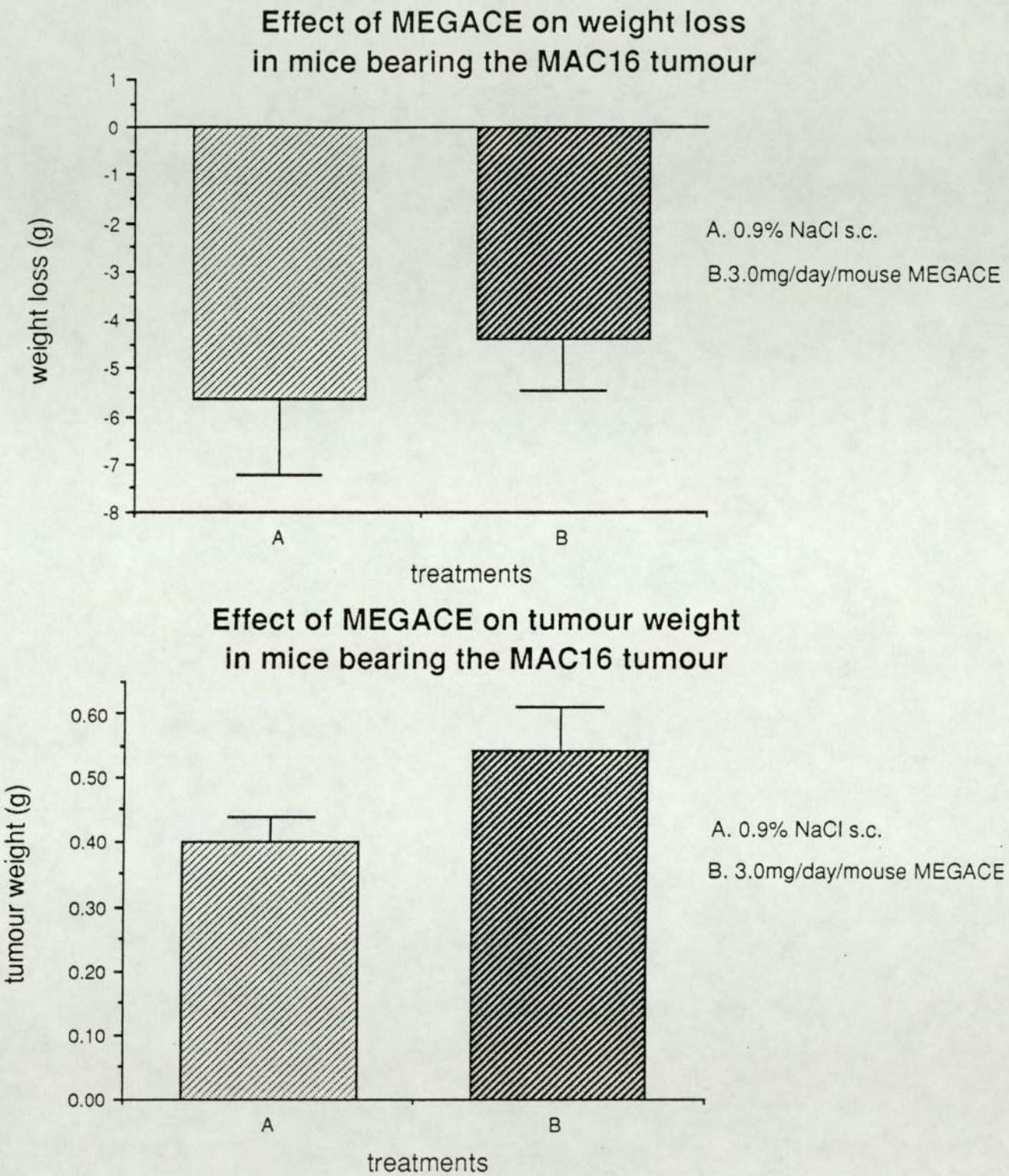
Lipolytic activity of plasma from MAC16 tumour bearing mice treated with insulin,MCT and/or fish oil



A. No treatment
B. 15U insulin s.c.
C. 20U insulin s.c
D. 5% fish oil diet
E. 25% fish oil diet
F. 80% MCT diet
G. 80% MCT diet + 3-hydroxybutyrate (30.0mM) in water
H. 25% fish oil + 55% MCT diet
I. 50% fish oil + 30% MCT diet

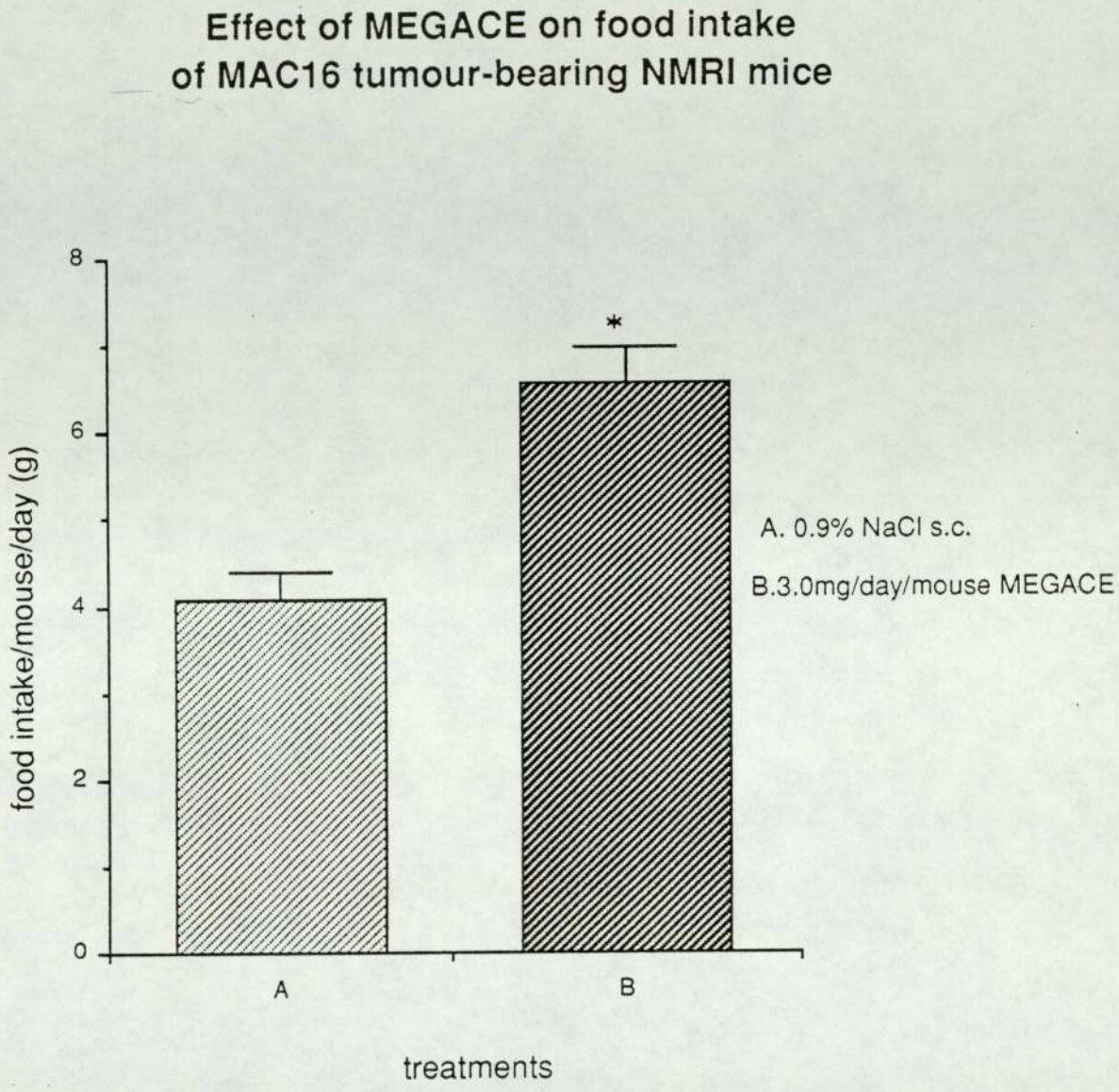
All treatments were carried out for 7/8 days. Results are expressed as mean±S.E.M. The number of animals analysed were 3 to 5. * significant difference = $p<0.05$ compared to control animals bearing the MAC16 tumour.

Figure 82



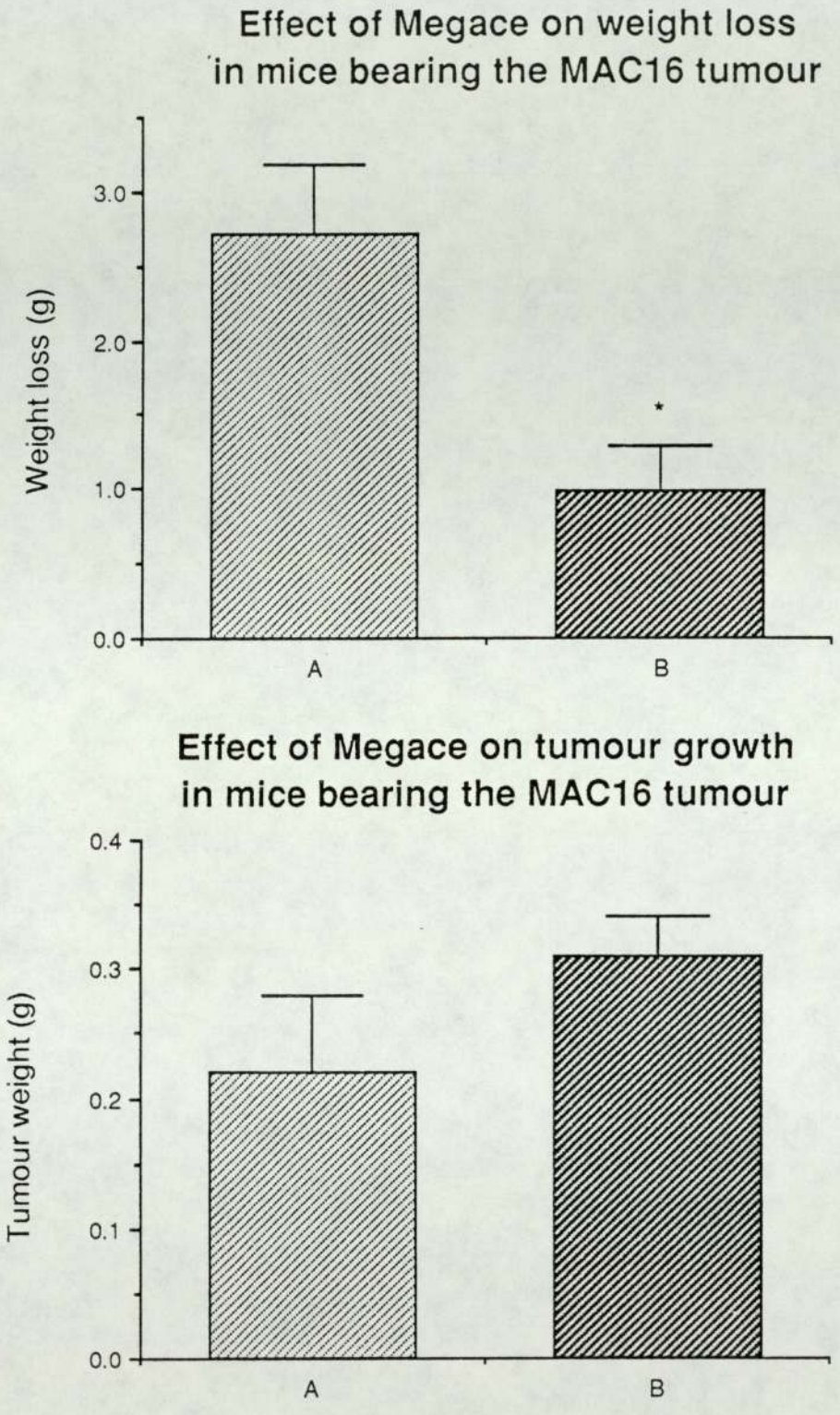
Results are expressed as Mean±S.E.M. Treatments were carried out for 8 days. Number of animals studied was 5 in each group.

Figure 83



The results are expressed as the Mean±S.E.M. Treatments were carried out for 8 days. Number of animals studied was 5 and the significant difference *$p<0.05$.

Figure 84

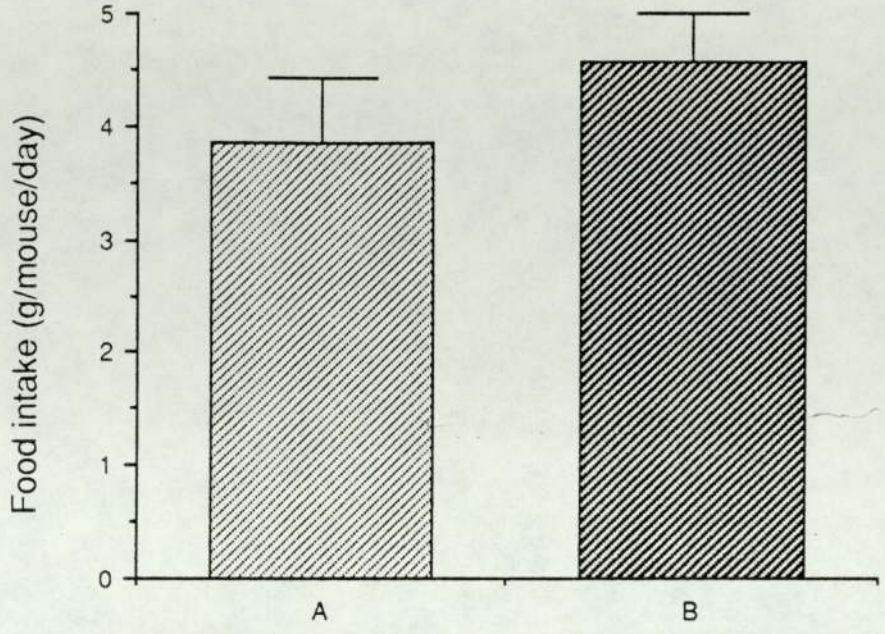


A. Control s.c 0.9% saline
B. s.c 6.0mg/mouse/day megace

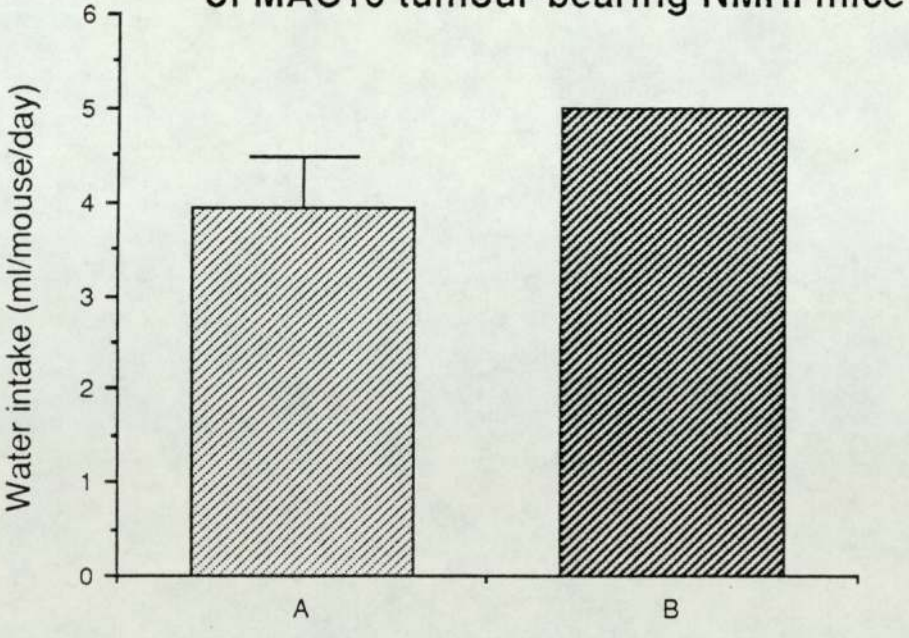
The results are expressed as the Mean ±S.E.M. the number of animals used per group were 5 to 10. Significant difference * $p<0.01$.

Figure 85

Effect of Megace on food intake of MAC16 tumour-bearing NMRI mice.



Effect of Megace on fluid intake of MAC16 tumour-bearing NMRI mice



A. Control s.c 0.9% saline
B. s.c. 6.0mg/mouse/day megace

The results are expressed as the Mean± S.E.M. The number of animals used per group were 5 to 10 .

bolite levels seen in the MAC16 tumour-bearing mice (table 19). One important feature was the significant increase in dry muscle weight. It was also noted that on dissection of the muscles they contained abnormally large amounts of fluid. This experiment was terminated after three days of treatment due to ulceration of tumours in the group recieving megestrol acetate. This needs to be studied further to identify if it is an effect of the the megace that the tumours prematurely ulcerated, again perhaps indicating a stimulation of tumour growth. A problem encounterd with megestrol acetate was its lack of solubility combined with the volume that could be administered subcutaneously to a mouse.

Table 19

Effect of megace on plasma metabolite levels in male NMRI mice bearing the MAC16 adenocarcinoma.

Tumour	Treatment	Glucose mM	FFA mM
MAC16	s.c 0.9% saline	4.45±0.37	0.36±0.08
MAC16	s.c 6.0mg/mouse/day Megace	4.16±0.27	0.32±0.05

Effect of megace on body composition of male NMRI mice bearing the MAC16 adenocarcinoma

Tumour	Treatment	Carcass fat (g)	Muscle dry weight (thigh+gastrocnemiu (g)
MAC16	s.c 0.9% saline	1.14±0.18	0.084±0.002
MAC16	s.c 6.0mg/mouse/day Megace	1.61±0.18	0.096±0.004*

The number of animals used per group 5 to 10.

Results are expressed as the Mean±S.E.M.

Significant difference *$p<0.05$.

Treatments were carried out for 3 days.

4.7.3 Discussion

From these *in vitro* and *in vivo* results a comparison of three diferent types of treatments for cachexia can be made.

Insulin has been shown to be effective in the prevention of weight loss induced by the MAC16 tumour. Insulin was also shown to be an effective inhibitor of the lipolytic and proteolytic factors produced by the MAC16 tumour, and this may be the reason for its anticachectic effects. Whether insulin reduces the effects of the MAC16 tumour produced catabolic factors by a direct inhibitory effect on the factors, or on their production, or an effect on the action of the factors remains unresolved. However, insulin inhibits lipase activation in adipose tissue (Bjorntorp 1966), and stimulates protein synthesis and inhibits protein degradation in isolated rat diaphragms (Fulks, Li and Goldberg 1975). There are two disadvantages to the use of insulin treatment in cachexia, its stimulation of tumour growth and its hypoglycaemic effect on the host. We have demonstrated an increase in tumour size after administration of exogenous insulin. These results oppose the work of Moley et al (1985) who found no stimulation of tumour growth in an anorexic model. Recent work from this group (Moley et al 1988), however, suggests an increased tumour weight in insulin treated animals. A number of tumours have been reported to grow faster in diabetic animals (Hissin and Hilf 1978, Sauer and Dauchy 1987). Insulin is a known stimulator of cell growth and has the capacity to increase substrate availability. This may be important in the insulin induced tumour growth reported

here. Occasional unexpected deaths occured during the insulin treatment presumably due to hypoglycaemic shock. A shortened survival of tumour bearing animals receiving long term insulin therapy has been reported (Moley et al 1985). Thus if insulin therapy was used in the treatment of cancer cachexia careful blood glucose monitoring of patients would be needed to avoid hypoglycaemic death.

Substitution of 80% of the calories as MCT was sucessful in the prevention of weight loss induced by the MAC16 tumour, in agreement with earlier work carried out in our laboratory (Tisdale et al 1987). The medium chain triglyceride diet increased the plasma level of ketone bodies and this is a method of inducing ketosis which is not observed in cachectic animals despite rapid depletion of adipose tissue (Bibby et el 1987). This treatment can be supplemented by addition of the ketone body 3-hydroxybutyrate to the drinking water. Most tumours have a high dependence on glycolysis and it is therefore theoretically possible to differentially feed the host and not the tumour. Buzby et al (1980) have shown that when fat was provided as the prime source of calories to a rat with a transplantable mammary carcinoma a more favourable host:tumour balance was obtained, when measured by the relative rates of growth of each. Another high fat diet significantly prolonged the survival of MCA-sarcoma bearing rats (Demetrakopoulos and Rosenthall 1982) and prevented anorexia in rats implanted with Walker 256 carcinosarcoma (Envione and Black 1983). However a high fat diet alone was not sufficient to prevent weight loss in NMRI mice since

replacement by LCT rather than MCT, which had no effect on plasma ketone body levels, had no effect on weight loss (Tisdale and Brennan, 1988). This was presumably due to the fact that in vitro 3-hydroxybutyrate was an effective inhibitor of the lipolytic and proteolytic factors produced by the MAC16 tumour. An advantage of this treatment was the concomitant reduction in tumour size. The circulatory levels of FFA were not elevated in these mice fed the high fat diets and this may indicate an increased peripheral tissue deposition of fat in these animals, which would lead to increased catabolism of fat by B-oxidation. It has been shown that a decreased oxygen tension was present in tumours coupled with a decreased enzymatic capacity to deal with ketone bodies (Tisdale and Brennan 1983). Thus utilisation of both FFA and ketone bodies by the tumour is minimal and so may account for the decrease in tumour size noted. Normal tissues would be able to utilize FFA and ketone bodies as an energy source and the excess ketone bodies would promote nitrogen conservation (Sherwin et al 1975). A number of clinical studies have been reported on high fat diets in cancer patients. Waterhouse and Nye (1961) showed a gain in body weight of patients with metastatic malignant disease when given a commercial fat emulsion by i.v infusion. These diets were nontoxic and well tolerated by the patients. Phinney et al (1983) reported that when normal human subjects were fed an 85% high fat diet it was well tolerated and there was no impairment of hepatic, renal, cardiac or haemopoietic function. Recently a clinical study was undertaken to determine the effect of

systemic ketosis in cachectic cancer patients. A normal diet was fed via a nasogastric tube to these patients for six days and then an isocaloric ketogenic diet containing 70% medium chain triglyceride and supplemented with 3-hydroxybutyrate (4mmol/kg/day) was fed for the next seven days. Before the start of nasogastric feeding the patients had lost approximately 32% of their pre-illness weight. The patients weight did not change significantly during the six days they were fed the normal diet. However, their mean weight increased by approximately 2kg during the period of feeding with the ketogenic diet. Thus the diet was sucessful in inducing weight gain in severly cachectic patients (Fearon et al 1988). No information on tumour growth rate was available from this study. Further clinical studies are needed to determine the efficacy of MCT on reduction of tumour size. Feeding by nasogastric tube is not practical on a large number of outpatients and if MCT is put into the clinic as a treatment of cachexia a more suitable method of administration is needed.

Diets enriched in fish oil have been shown to reduce both the growth rate and the weight loss produced by the MAC16 adenocarcinoma. Replacement of 50% of the calories by fish oil was required for optimal activity but the diets were well tolerated and no toxicity was observed. The effects appear to be specific for the polyunsaturated fatty acids found in fish oil as animals fed diets with up to 50% of the calories replaced by gamma linoleic acid had no effect on tumour size or weight loss (unpublished results J.Dhesi). As stated before fish oil consists of

ecoisapentaenoic acid (EPA) and docosahexaenoic acid (DCH). In vitro EPA significantly inhibits the catabolic factors produced by the MAC16 adenocarcinoma. Antitumour activity of EPA has recently been demonstrated in patients with metastatic breast cancer (Holroyde et al 1988). Substitution of EPA for arachidonic acid in membrane lipids leads to competition for the cyclooxygenase and an inhibition of prostaglandin synthesis (Karmali et al 1985), which may account for the inhibition of mammary tumour growth. The growth of both the MAC13 and MAC16 adenocarcinomas was inhibited by EPA oil in vivo and the lipolytic activity of these tumours was inhibited by EPA in vitro. The inhibition of lipolysis in vitro was reversed by prior addition of carnitine. Carnitine is a fatty acid transporter so it is possible that the factor is necessary to provide transport for the fatty acids across the cell membrane into the cell. Inhibition of this factor also reduces glucose uptake and increases lactate production of the cells. Therefore, it is possible that the lipolytic factors we have identified in the MAC13 and MAC16 adenocarcinomas are necessary for tumour growth. Fish oil has a good anticachectic and antitumour effect on the MAC16 adenocarcinoma. At the moment clinical studies of fish oil and pure EPA in cachectic cancer patients are being considered. It has been shown in male volunteers that the absorption of EPA from fish oil was significantly improved from 69% to 90% by coinjestion with a high fat meal (Lawson and Hughes 1988). Thus clinical studies on the effects of EPA need to be designed to account for this variation in

absorption with meal fat content.

The MAC16 tumour is generally resistant to conventional cytotoxic agents but it was highly responsive to the investigational agent flavone acetic acid (FAA) (Bibby et al 1988). Flavone acetic acid (FAA) is a novel antitumour agent under early clinical investigation in Europe and the U.S.A. This compound has a significant antitumour effect in solid murine tumours (Bibby et al 1987a). Bibby et al (1987c) have demonstrated that tumour vasculature may be important in the action of FAA as the antitumour effects are more marked as the vascular composition of the tumour increases. It was noted that the antitumour effects were accompanied by a control of the tumour associated cachexia. Further experiments on the metabolic effects of FAA on the tumour and host are needed. Megestrol acetate produced a reduction in weight loss induced by the MAC16 adenocarcinoma. However, this was concomittant with an increase in food intake and a stimulation of tumour growth. These latter properties are not desirable in a treatment for cachexia. It is possible that the megestrol acetate effects are due to its steroidal nature. Its anabolic actions would cause an increase in appetite and weight gain. The increase in muscle weight reported and fluid retention are common side effects of progesterones. Thus the anti-cachectic properties of Megace may be due to its progesterone-like side effects. The stimulation of tumour growth could be due to anabolic factors increasing substrate availability as with insulin. In aggreement , it has been noted in the ongoing clinical study of megestrol acetate

that there may be a slight stimulation of tumour growth in one patient and no regression of tumours has been reported (Dr. C. Barton, personal communication).

To summarise these results the dietary changes seem to be the better treatments of cachexia. As both MCT and fish oil reduce weight loss with the added advantage of an antitumour effect. These diets are well tolerated and non toxic to the host. Insulin and megace both have a major disadvantage as they increase the size of the tumour. This is obviously undesirable in an anticachectic treatment (table 20).

Table 20

Substance	Type of treatment	Reduce weight loss	Effect on tumour size	Effect on food intake
Megace	drug	yes	increased	increased
Insulin	drug	yes	increased	none
3-hydroxy butyrate	ketone body	yes	none	none
MCT	fat diet	yes	decreased	none
Fish oil	dietary	yes	decreased	none

Prevention of the cachectic effect of the tumour was greater with fish oil than observed with the ketogenic diet. Further clinical investigations into the effects of fish oil and/or pure EPA in the treatment of cachexia are warranted. It is interesting that all of these treatments can be linked *in vitro* to inhibition of the MAC16 tumour produced lipolytic and proteolytic factors. This further supports the hypothesis that these factors may be responsible for the cachexia observed in the host.

SECTION 5: GENERAL DISCUSSION

5.0 General discussion

Evidence presented in section 4 shows that the MAC16 adenocarcinoma is a good model for the use in the study of cancer cachexia. It is analogous to the situation seen in cancer patients where host weight loss occurs with only a small tumour burden without an effect on food or fluid intake. The MAC16 adenocarcinoma has been shown to produce circulatory catabolic factors that have lipolytic and proteolytic activities in the host. On characterisation of the proteolytic factor it was found to be a trypsin-like serine protease analogous to the serine protease guanidinobenzoatase thought to be involved in tumour metastases (Steven, 1988). With further purification and charcterisation of these catabolic factors various lipolytic and proteolytic factors were identified. The proteolytic factors (molecular weight > 150k daltons) were distinguishable from the lipolytic factors which appeared related and had molecular weights of approximately 3k, 1.5k and 0.7k daltons.

The possible identity of these factors is at the present unknown. They are thought to be peptides with possibly phosphate groups attached. The larger of the peptides may be a complex involving the smaller peptide. A metal ion may be involved in this complex as preliminary experiments have shown that EDTA can breakdown the 3k material to the 0.7k dalton factor (T. Mcdevitt, unpublished results). It is further hypothesised that the lipolytic factors could be small peptides containing a

common functional molecular grouping directly related to or responsible for their lipolytic activity.

These factors may cause their cachectic actions via the adenylate cyclase second messenger system.

Lipolytic factors of the same molecular weights as identified in the MAC16 adenocarcinoma were isolated in small amounts in other non-cachectic tumour models and in the body fluids of tumour-bearing animals and cancer patients. These factors were not present in healthy individuals before or after a 24 hour fast or in patients with other weight losing conditions.

All treatments for cachexia studied in the MAC16 adenocarcinoma can be linked in vitro to inhibition of these catabolic factors that have been isolated. Thus the hypothesis is put forward that these factors may be responsible for the cachexia the tumour confers on its host.

To further substantiate this hypothesis experiments have been performed whereby the partially purified lipolytic factor was injected intra-peritoneally into non tumour-bearing NMRI mice. The mice did experience significant weight loss without a significant reduction in food or fluid intake compared with no significant weight loss seen in control animals injected with 0.9% saline (H. Bailey, unpublished results), although overnight, after feeding there was some restoration of weight loss possibly due to the resynthesis of triglycerides. Non cachectic animals bearing the MAC16 tumour and animals bearing the non cachexia-inducing tumour the MAC13 adenocarcinoma were injected with partially purified factor. An even greater

weight loss was seen again with no effect on food or water intake. Thus the tumour may be acting as a sink for the free fatty acids broken down from triglycerides (H. Bailey, unpublished results).

These factors appear to be present in all tumours in both experimental and human models that have been examined in these laboratories. The amount of factor present depends on whether the tumour confers cachexia on its host. If this factor is present in all tumours it most probably performs an essential role in tumour growth. It is possible that the factor is used by the tumour to supply its lipid requirements for membrane formation and/or to fuel its energy supply. However, why the tumours need such a vast quantity of lipids from the host is unknown. Preliminary results suggest that the lipolytic factors may stimulate the growth of the MAC16 cell line in culture at low concentrations but inhibit the growth of cells at high concentrations (T. Mcdevitt, unpublished results). Thus once isolated the lipolytic factors may provide a specific target for antitumour therapy. This would be a novel target in that it would preferentially inhibit tumour growth with a concomitant treatment of the patients cachexia.

The aim of future experiments are to completely purify the lipolytic factors and to elucidate their structure. This may be achieved by using amino acid analysis and/or FAB mass spectrometry. As the factors are so small then it would be easy to synthesize them and prepare monoclonal antibodies . Once this has been achieved the pharmacokinetic properties must be studied.

After isolation, sequencing and synthesis of these materials many potential uses can be advocated. The active lipolytic factor may be treated as a tumour marker and be used for diagnostic purposes, to detect the presence of a tumour in a patient at an early stage before any clinical diagnosis has been made. It could also be used to monitor the progress of the tumour response after treatment.

Monoclonal antibodies may be employed as part of a diagnostic kit. However , a more important use of monoclonal antibodies will be as inhibitors or antagonists to the active lipolytic factor in human cancer patients. They will constitute a therapeutic agent for treating and suppressing the symptoms of cachexia. Specific inhibitors may be synthesised directly from the known structure of the factors and also used in the treatment of cachexia.

These factors may in addition have a potential therapeutic value for use in the controlled treatment of obesity in mammals, including humans. The pure active substance would be needed to be made up into pharmaceutical formulations for administration in any suitable manner (e.g. parenterally or orally).

Obviously in all these cases a lot more work is needed before these ideas can be seriously entertained.

Clinically cachexia is a serious problem and the identification of tumour produced factors that may contribute to this wasting is an intriguing finding. Solid tumours are fairly unresponsive to present chemotherapeutic regimens and so the advancement of knowledge of the

mechanism of tumour cell growth may help improve this situation.

If these factors are essential for tumour growth, then the inhibition of their production and/or action may not only reverse cachexia but also provide a novel approach to the treatment of neoplasms.

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APPENDICES

APPENDIX 1

RAT & MOUSE BREEDING DIET 10mm [422]

Calculated Analysis

Nutritional Information

Crude Oil	%	4.12
Crude Protein	%	20.29
Crude Fibre	%	4.13
Total Ash	%	8.99
Neutral Detergent Fibre	%	18.8
Hemicellulose	%	13.1
Acid Detergent Fibre	%	5.7
Starch	%	36.11
Sugars	%	3.04
Gross Energy	Mj/kg	15.9988
Digestible Energy	MJ/kg	13.3073
Metabolisable Energy	Mj/kg	11.8298
Essential Fatty Acids	%	1.4462
D.C.P	%	16.6995
Lysine	%	0.9826
Methionine	%	0.3521
Methionine & Cystine	%	0.6255
Threonine	%	0.6469
Tryptophan	%	0.1975
Argenine	%	1.1844
Calcium	%	1.7232
Phosphorus	%	1.0940
Available Phosphorus	%	0.8555
Sodium	%	0.2770
Potassium	%	0.5616
Salt (NaCl)	%	0.6083
Magnesium	%	0.05

Vitamins & Trace Elements Added by Supplementation

Iron	mg/kg	50.00
Copper	mg/kg	5.00
Manganese	mg/kg	50.00
Zinc	mg/kg	15.00
Iodine	mg/kg	0.50
Cobalt	mg/kg	-
Selenium	mcg/kg	-
Vitamin A	IU/kg	15000.00
Vitamin D	IU/kg	2000.00
Vitamin E	mg/kg	70.00
Vitamin K	mg/kg	10.00
Vitamin B1	mg/kg	5.00
Vitamin B2	mg/kg	6.00
Vitamin B6	mg/kg	7.50
Vitamin B12	mcg/kg	7.50
Folic Acid	mg/kg	2.00
Nicotinic Acid	mg/kg	12.00
Pantothenic Acid	mg/kg	15.00
Choline Chloride	mg/kg	1000.00
Biotin	mcg/kg	200.00
Vitamin C	mg/kg	-

Notes

1. The specifications quoted above are those at the time of printing and are only intended as a guide. Changes in conditions will alter, thus effecting the values given above. When this occurs it will be recorded on the bag label ammended extended analysis sheet will be issued.
2. Values in the left hand set of figures are total calculated values.
3. Figures for trace elements and vitamins are the amounts added by supplementation.
4. 1 MJ = 239.005 Kcal
5. 1 IU of Vitamin A = 0.344 mcg pure Vitamin A Acetate.
6. 1 IU of Vitamin D = 0.025 mcg pure Vitamin D2/D3
7. 1 IU of Vitamin E = 1mg DL Alpha Tocopherol Acetate.
8. Further analytical information can be provided for batches as an extended analysis.

APPENDIX 2

List of publications

ABSTRACTS:

1. R.A. Brennan, S.A. Beck, and M.J. Tisdale (1987)
   Cachectic factor(s) produced by the MAC16 adenocarcinoma.
   Joint meeting of the 28th British Association for Cancer Research and the 2nd Association of Cancer Physicians, Newcastle, U.K.

2. S.A. Beck and M.J. Tisdale (March 1988)
   Circulatory lipolytic factors in cancer cachexia.
   Joint meeting of the 29th British Association for Cancer Research and the 3rd Association of Cancer Physicians, Norwich, U.K.

3. M.J. Tisdale and S.A. Beck (May 1988)
   Cachectic factors produced by human and animal tumours.
   79th annual meeting of the American Association for Cancer Research, New Orleans, U.S.A.

4. S.A. Beck and M.J. Tisdale (1989)
   Effect of insulin and ketone bodies on tumour growth and host body weight in a cachexia model.
   Joint meeting of the 30th British Association for Cancer Research and the 4th Association of Cancer Physicians, Glasgow, U.K.

PAPERS:

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

2. S.M. Mahony, S.A. Beck and M.J. Tisdale (1988)
   Comparison of weight loss induced by a recombinant tumour necrosis factor with that produced by a cachexia-inducing tumour.
   British Journal of Cancer, 57, 385-389.

3. S.A. Beck and M.J. Tisdale (March 1989)
   Effect of insulin on weight loss and tumour growth in a cachexia model.
   British journal of cancer, in press.

4. S.A. Beck and M.J. Tisdale (March 1989)
   Nitrogen excretion in cancer cachexia and its modification by a high fat diet.
   Cancer Research, in press.

PATENT:

Biologically active preparations characterised by catabolic activity associated with cachexia-inducing tumours production and uses thereo

Cachectic factor(s) produced by the MAC 16 adenocarcinoma

R.A. Brennan, S.A. Beck & M.J. Tisdale

CRC Experimental Chemotherapy Group, Department of Pharmaceutical Sciences, Aston University, Birmingham B4 7ET, UK.

The MAC 16 is a transplantable colon adenocarcinoma which produces extensive weight loss in tumour-bearing animals without a reduction in food or water intake. In males a 0.6 g tumour will produce 33% loss of body weight within 35 days of tumour transplantation. Body composition analysis shows a progressive decrease in adipose tissue and muscle mass without a change in body water. While plasma glutamine levels are elevated 25% in tumour-bearing animals the plasma concentrations of most other amino acids including glycine are reduced by 30–40% and thus the situation differs from chronic malnutrition. Cell-free extracts of the MAC 16 tumour cause a release of free fatty acids (FFA) from mouse fat pads while extracts from two colon carcinomas, which do not produce cachexia in recipient animals, MAC 13 and MAC15A, have no effect on FFA release. The FFA releasing activity of the MAC 16 tumour is dramatically reduced after acid or heat treatment. Cell-free extracts of the MAC 16 tumour also cause an enhanced release of amino acids from mouse diaphragm, while extracts from MAC 15A do not. These results suggest that the cachexia produced by the MAC 16 tumour may be due to the presence of a tumour-associated catabolic factor.

Circulatory lipolytic factors in cancer cachexia.

S.A. Beck and M.J. Tisdale, CRC Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET

We have utilised a transplantable colon adenocarcinoma of the mouse (MAC16) as a model of human cancer cachexia. This tumour produces extensive weight loss in the host at small tumour burdens (about 2% of the total body weight) and without a reduction in either food or water intake. Weight loss is associated with a decrease in both carcass fat and muscle mass which is directly proportional to the weight of the tumour. Weight loss in this murine model has been correlated with the production by the tumour of both lipolytic and proteolytic factors, which are present in the circulation. Both factors respond to normal metabolic control and are inhibited by insulin and 3-hydroxybutyrate. Using DEAE cellulose chromatography and gel filtration the lipolytic and proteolytic factors have been shown to be distinct and to be separable into a number of fractions. Serum from cancer patients with extensive weight loss shows an elevated lipolytic activity when compared with normal subjects. Fractionation of serum from cancer patients using ion exchange chromatography shows evidence of lipolytic activity which elutes at the same ionic strength as that produced by the MAC16 tumour, while serum from normal control subjects contains no peaks of lipolytic activity in the same position. This raises the possibility that cachexia in both animals and humans may be due to circulatory lipolytic factors.

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Cachectic Factors produced by Human and Animal Tumours. M.J. Tisdale and S.A. Beck, Cancer Research Campaign Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4, 7ET, U.K.

A transplantable mouse colon adenocarcinoma (MAC16) produces a progressive decrease in the carcass weight of the host, which is directly proportional to the tumour burden, and is associated with a decrease in both carcass fat and lean body mass. In males weight loss reaches 33% when the tumour represents just 2% of the total body mass. The weight loss is not associated with a decrease in either food or water intake or with the production of tumour necrosis factor.

We have measured glycerol release from mouse epididymal adipocytes and amino acid release from mouse diaphragm to investigate the possibility of lipolytic and proteolytic factors which may be responsible for the cachexia. This has shown the presence of tumour associated lipolytic and proteolytic factors which are released into the circulation. Insulin and 3-hydroxybutyrate both suppress the lipolytic and proteolytic activity of tumour extracts. Using DEAE cellulose and exclusion chromatography the lipolytic and proteolytic factors have been shown to be distinct and resolvable into a number of fractions. The lipolytic factor appears to be a polypeptide of MW 3KD. Sera from patients with cancer cachexia have an elevated lipolytic activity when compared with controls. Using DEAE cellulose chromatography it can be shown that the lipolytic factors produced by cancer patients elute at the same ionic strength as those produced by the MAC16 tumour and that moreover normal serum contains no corresponding fractions of lipolytic activity. These results suggest that cachexia in both experimental animals and humans may be due to the production of circulatory catabolic factors by the tumour.

EFFECT OF INSULIN AND KETONE BODIES ON TUMOUR GROWTH AND HOST BODY WEIGHT IN A CACHEXIA MODEL. S.A. Beck and M.J. Tisdale. CRC Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET.

We have utilized the MAC16 adenocarcinoma of the mouse colon as an experimental model of human cachexia where weight loss occurs without a reduction in food or water intake. Weight loss produced by the MAC16 tumour is characterized by a loss of body fat and muscle dry weight, which increases in direct proportion to the tumour burden, and is associated with the presence of circulatory catabolic factors, which degrade host muscle and adipose tissue in vitro. Both insulin and 3-hydroxybutyrate inhibit the lipolytic and proteolytic activity in vitro, and thus a comparison has been made between the effects of daily insulin injections and a ketogenic diet on weight loss and tumour growth in the MAC16 model. Weight loss was significantly reduced by both a ketogenic diet (80% of calories supplied as medium chain triglyceride) and by administration of 20U insulin kg^{-1} day^{-1} without an alteration in either water consumption or total calorie intake. At the end of the experiment the tumour weight in animals fed a ketogenic diet was significantly lower than those consuming the normal laboratory diet, while in animals treated with insulin the tumour weight was 50% greater than controls. The enhancement of tumour weight by insulin was prevented by the concurrent administration of sodium 3-hydroxybutyrate. Both carcass fat and muscle dry weight were elevated in animals fed the ketogenic diet or administered insulin when compared with controls. These results suggest that both insulin and ketone bodies are effective in prevention of weight loss in cancer cachexia, but that insulin administration may be associated with an enhanced tumour growth rate.

[CANCER RESEARCH 47, 5919-5923, November 15, 1987]

Production of Lipolytic and Proteolytic Factors by a Murine Tumor-producing Cachexia in the Host[1]

Susan A. Beck[2] and Michael J. Tisdale

CRC Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, United Kingdom

ABSTRACT

Animals given transplants of the MAC16 colon adenocarcinoma show a progressive decrease in carcass weight as the tumor size increases without a reduction in either fluid or caloric intake when compared with non-tumor-bearing controls. There is a decrease in both carcass fat and muscle mass which is directly proportional to the weight of the tumor. In male animals weight loss occurs when the tumor mass comprises more than 0.3% of the body weight and reaches 30% when the tumor represents 3% of the body weight.

There is evidence for the production by the tumor of both lipolytic and proteolytic factors, which may be responsible for the cachexia, since two related mouse adenocarcinomas, which do not produce weight loss, have little lipolytic or proteolytic activity. The lipolytic factor is nondialyzable and is destroyed by both heat and acid. Both insulin and 3-hydroxybutyrate suppress the lipolytic activity of the tumor extract. The MAC16 tumor also contains a serine protease, the activity of which is also completely abolished by insulin and 3-hydroxybutyrate. Animals bearing the MAC16 tumor have an elevated plasma lipolytic and proteolytic activity when compared with non-tumor-bearing controls, suggesting a peripheral effect of the tumor products. The catabolic factors elaborated by the MAC16 adenocarcinoma may be responsible for the loss of both the fat and nonfat carcass mass, but they do respond to normal metabolic controls.

INTRODUCTION

Cancer cachexia involves a massive loss of body weight, with extensive breakdown of both body fat and skeletal muscle, often, although not always, accompanied by anorexia (1). Many patients display a significant weight loss as their first symptom of neoplasia (2) with a small primary tumor often less than 0.01% of the total body weight (3). It is, therefore, unlikely that the tumor simply competes with the host for available nutrients.

Theologides (4) has proposed that peptides, oligonucleotides, or other metabolites produced by cancer cells are responsible for alterations in the metabolic patterns of the host. There is some evidence for the role of circulatory factors in the etiology of cancer anorexia/cachexia. A parabiotic preparation of a rat sarcoma, for which there was no evidence of metastases or endocrine function, provided evidence for the humoral transmission of an anorectic/cachectic factor (5). A lipid-mobilizing substance (toxohormone-L) has been isolated from tumor extracts and body fluids of patients and animals with tumors (6). Injection of toxohormone-L into the lateral ventricle of rats significantly suppressed food and water intake (7). Further evidence for a lipid-mobilizing factor was found in the serum of tumor-bearing AKR mice (8), although this has not been characterized. A substance immunochemically cross-reactive with insulin is produced by B16 melanoma cells growing in diabetic and nondiabetic mice and is correlated with a decreased concentration of circulatory glucose and an elevated concentration of growth hormone in the blood (9). Recently a macrophage product, cachectin (tumor necrosis factor), which inhibits lipoprotein lipase activity in peripheral tissues, has been suggested to orchestrate the complex metabolic changes that lead to cachexia (10).

As an experimental model of cachexia we have utilized a transplantable colon adenocarcinoma (MAC16) passaged in NMR1 mice (11). This tumor produces extensive host weight loss at tumor burdens less than 1% of the host weight and without a drop in caloric intake. Since host weight loss occurs with such a small tumor mass it is unlikely that the tumor simply competes with the host for available nutrients. This raises the possibility of the production of catabolic factors by the tumor, which act to degrade host muscle and fat stores.

MATERIALS AND METHODS

Animals. Pure strain male BALB/c and NMRI mice were purchased from Banting and Kingman, Hull, United Kingdom, and were fed a rat and mouse breeding diet (Pilsbury, Birmingham, West Midlands, United Kingdom) and water *ad libitum*. Fragments of the MAC16 tumor (1 x 2 mm) were implanted in the flanks of NMRI mice by means of a trocar as described (11). The doubling time of this tumor is about 6 days (11). Tumors were removed 14 to 42 days after transplantation when the tumor weighed between 0.1 and 0.6 g. Tumors were removed before weight loss exceeded 40%. The tumors were homogenized at 4°C in Krebs-Ringer bicarbonate buffer, pH 7.6 (1 ml/0.1 g of tumor), and centrifuged for 10 min at 3000 rpm to remove debris. The supernatant was used for the determination of lipolytic and proteolytic activity. Aliquots were also stored at −20°C without loss of activity, although repeated freezing and thawing destroyed the effect. The protein content of tumor extracts was determined by the method of Lowry *et al.* (12). Plasma samples were obtained from freely fed animals and blood was removed between 10 and 11 a.m.

Fragments of the MAC13 tumor (obtained from Dr. J. Double, University of Bradford, United Kingdom) were also implanted in the flank of male NMRI mice. This tumor has a doubling time of 7 days and there is no weight loss during the growth of the tumor. MAC15A cells were grown in tissue culture in RPMI 1640 medium under an atmosphere of 5% CO_2 in air. This tumor is derived from an ascites tumor which grows in NMRI mice without weight loss and was obtained from Dr. M. Bibby, University of Bradford, United Kingdom. MAC15A cells can be transplanted into mice from tissue culture to produce a tumor which is the same as the original. Blood was removed from animals by cardiac puncture under anesthesia using a mixture of halothane, oxygen, and nitrous oxide by way of a heparinized syringe.

Body Composition Analysis. The gastrocnemius and thigh muscles from the left hind leg of the carcass were carefully dissected out and weighed, together with the tumor. Each carcass plus muscles were heated at 80°C until a constant weight was achieved. Carcasses were then reweighed and the water content was determined from the difference between the wet and dry weights. Total carcass fat was determined by the method of Lundholm *et al.* (13).

Determination of Lipolytic Activity. Male BALB/c mice were killed by cervical dislocation and their epididymal adipose tissue was quickly removed and minced in Krebs-Ringer bicarbonate buffer, pH 7.6. Approximately 50–100 mg of the adipose tissue were incubated with tumor supernatants in a total volume of 0.25 ml of the Krebs-Ringer buffer. Controls containing adipose tissue and buffer alone were included in each experiment and the spontaneous release of FFA[3] was

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[1] This work has been supported by a grant from the Cancer Research Campaign.

[2] Recipient of a research studentship from the Cancer Research Campaign.

[3] The abbreviation used is: FFA, free fatty acids.

subtracted from the values obtained with tumor present. Incubations were continued for up to 2 h at 37°C and the FFA concentrations in the cell-free supernatants were determined immediately using a Wako NEFA C kit (Alpha Laboratories, Ltd., Hampshire, United Kingdom).

Determination of Proteolytic Activity. Male BALB/c mice were killed by cervical dislocation and the diaphragms were carefully dissected out, blotted, cut in half, and weighed; each half was placed in a stoppered vial containing 0.75 ml Krebs-Ringer bicarbonate buffer and gassed for 20 s with 5% CO_2 in air. Preincubations were carried out for 30 min at 37°C, and the diaphragms were then blotted and transferred to clean vials containing tumor extract and Krebs-Ringer bicarbonate buffer in a total volume of 0.75 ml. The vials were gassed and incubated for a further 2 h at 37°C. Incubations were terminated by mixing 0.5 ml of the assay mixture with 0.125 ml of cold 50% trichloroacetic acid, mixing, and centrifuging for 10 min at 3000 rpm. The supernatants were neutralized with 1 N NaOH and 200 μl of the neutralized sample were mixed with 1 ml of ninhydrin reagent and held in a boiling water bath for 20 min; after dilution to 5 ml with *n*-propanolyl alcohol:water (1:1), the concentration of amino acids was determined spectrophotometrically at 570 nm. The spontaneous release of amino acids from the diaphragms in the absence of any additions was subtracted from the final readings.

To determine the proteolytic activity of plasma samples 50 μl of plasma were added to 0.7 ml of Krebs-Ringer bicarbonate buffer containing 0.5 mM cycloheximide to inhibit protein synthesis and incubated with the half-diaphragms for 2 h as above. Controls containing no diaphragm were subtracted from the final readings to eliminate the free amino acids present in plasma samples.

Statistical Analyses. Statistical evaluations were accomplished with the use of analysis-of-variance techniques, with individual means compared by Student's *t* tests.

RESULTS

Animals given transplants of the MAC16 adenocarcinoma show a progressive decrease in carcass weight as the tumor size increases (Fig. 1). The average food intake in tumor-bearing animals [15.1 ± 0.6 (SE) kcal/day] is not significantly different from that in non-tumor bearing animals [14.9 ± 0.9 kcal/day]. Also tumor-bearing animals have the same water intake (4.6 ± 0.27 ml/day) as do controls (4.8 ± 0.16 ml/day). In male mice the tumor must reach a weight of 0.1 g in a 30-g animal before any weight loss occurs, but thereafter weight loss is directly proportional to tumor weight (correlation coefficient, 0.91). Weight loss is associated with a decrease in both carcass fat (Fig. 2) and muscle dry weight (Fig. 3) which again decrease in direct proportion to the weight of the tumor. Loss of body fat exceeds that of muscle by about 13 times for a given weight of tumor.

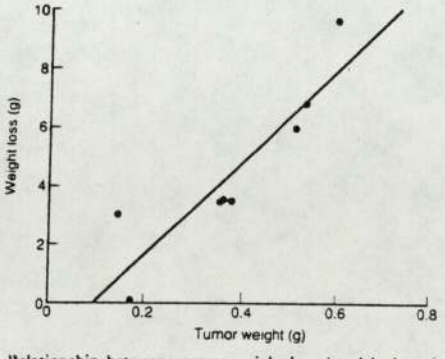

Fig. 1. Relationship between carcass weight loss (total body weight minus tumor weight) produced by the MAC16 tumor and tumor weight. The results were fitted to a linear model by a least squares analysis ($r = -0.91$). Each *point* represents the results from an individual animal.

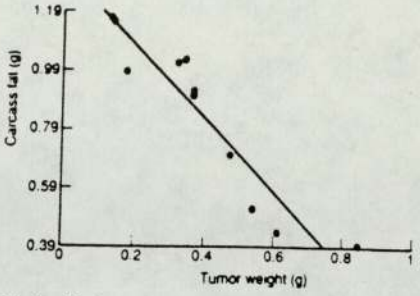

Fig. 2. Relationship between total body fat (excluding tumor) and weight of the MAC16 tumor. The results were fitted to a linear model by means of a least squares analysis ($r = -0.93$). Each *point* represents the results from an individual animal.

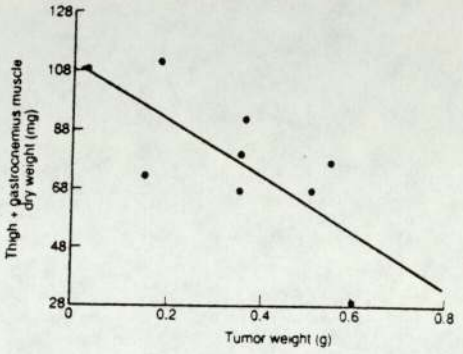

Fig. 3. Relationship between thigh and gastrocnemius muscle dry weight and weight of the MAC16 tumor. The results were fitted to a linear model by means of a least squares analysis ($r = -0.65$).

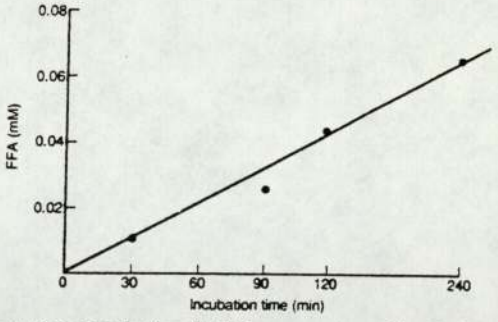

Fig. 4. Rate of production of FFA from mouse fat pads by MAC16 tumor homogenate. A cell-free preparation of the MAC16 tumor was prepared as described in "Materials and Methods," and a fraction (2.6 mg of protein) was incubated with 200 mg of epididymal adipose tissue, and the rate of release of FFA was determined as described in "Materials and Methods."

The direct correlation between body compartment sizes and the tumor burden suggests the possibility that tumor-derived products are responsible for the cachexia. To investigate the possible production of lipolytic factors, crude tumor supernatants were incubated with epididymal adipose tissue and the release of FFA into the medium was assayed. Extracts of the MAC16 tumor caused an enhanced release of FFA, which increased linearly with the time up to 4 h (Fig. 4). As a control cell-free extracts were prepared from two other colon adenocarcinomas, MAC13 and MAC15A, neither of which produces cachexia in recipient animals. The rate of release of FFA by

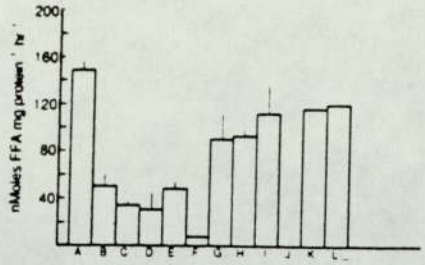

Fig. 5. Rate of release of FFA from mouse epididymal adipose tissue by tumor homogenates. *A*, MAC16 tumor; *B*, MAC15A tumor homogenate; *C*, MAC13 tumor homogenate; *D*, MAC16 tumor homogenate plus 10 units insulin per assay; *E*, MAC16 tumor homogenate plus 8 mM sodium 3-hydroxybutyrate; *F*, MAC16 tumor homogenate plus 10 units insulin plus 8 mM sodium 3-hydroxybutyrate; *G*, MAC16 tumor homogenate plus 1 mM indomethacin; *H*, MAC16 tumor homogenate plus 1 mM propranalol; *I*, MAC16 tumor homogenate plus 0.5 mM phenylmethylsulfonyl fluoride; *J*, 15.4 mM NaCl; *K*, MAC16 tumor homogenate plus 15.4 mM NaCl; *L*, MAC16 tumor homogenate plus 1 mg/ml trypsin inhibitor. The results are expressed as means of 3–13 determinations; *bars*, SE. *B*, *C*, *D*, *E*, *F*, $P < 0.001$ from *A* by Student's *t* test.

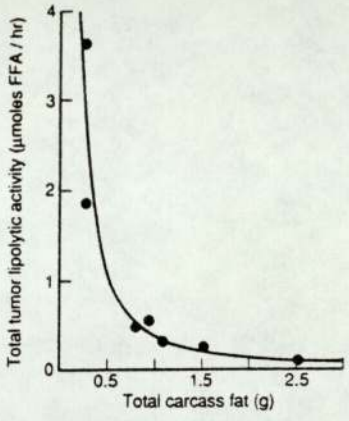

Fig. 6. Regression analysis of the relationship between the total tumor content of lipolytic activity and the total carcass fat. The results fit a multiplicative model ($r = 0.975$).

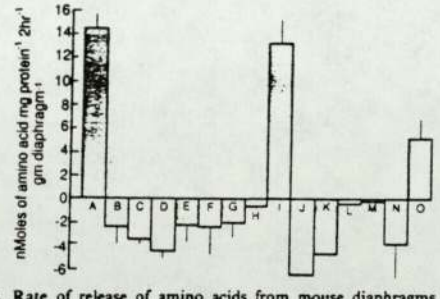

Fig. 7. Rate of release of amino acids from mouse diaphragms by tumor homogenates. *A*, MAC16 tumor; *B*, MAC15A tumor; *C*, MAC13 tumor; *D*, 10 units insulin per assay; *E*, MAC16 tumor homogenate plus 10 units insulin; *F*, 8 mM sodium 3-hydroxybutyrate; *G*, MAC16 tumor homogenate plus 8 mM sodium 3-hydroxybutyrate; *H*, 10.4 mM NaCl; *I*, MAC16 tumor homogenate plus 10 mM sodium chloride; *J*, 8 mM sodium 3-hydroxybutyrate plus 10 units insulin; *K*, MAC16 tumor homogenate plus 10 units insulin plus 8 mM sodium 3-hydroxybutyrate; *L*, 0.5 mM phenylmethylsulfonyl fluoride; *M*, MAC16 tumor homogenate plus 0.5 mM phenylmethylsulfonyl fluoride; *N*, trypsin inhibitor (1 mg/ml); *O*, MAC16 tumor homogenate plus 1 mg/ml trypsin inhibitor. The results are expressed as means of 3–13 determinations; *bars*, SE. *B*, *C*, *D*, *E*, *F*, *G*, *H*, *L*, *M*, *N*, $p < 0.001$ from A. *O*, $p < 0.05$ from *A*. *B*, *C*, *D*, *F*, *J*, *N*, $p < 0.001$ from control by Student's *t* test.

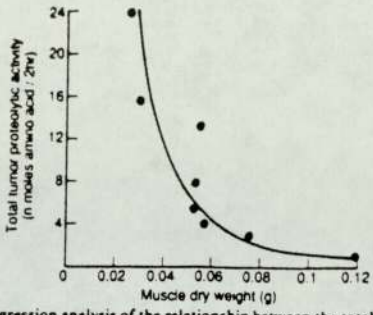

Fig. 8. Regression analysis of the relationship between the total tumor content of proteolytic activity and the thigh and gastrocnemius muscle dry weights. The results fit a multiplicative model ($r = 0.932$).

these tumor extracts was less than one-third of that produced by the MAC16 tumor (Fig. 5). The lipolytic activity associated with the MAC16 tumor was nondialyzable and was destroyed by both heat and acid (Fig. 5). Neither propanolol nor indomethacin at concentrations of up to 1 mM in the incubation assay had any significant effect on the lipolytic activity of the MAC16 tumor extract. Inhibition of proteolysis by the trypsin inhibitor or phenylmethylsulfonyl fluoride did not reduce lipolysis by tumor extracts. Lipolysis induced by the MAC16 tumor was significantly reduced by both insulin and 3-hydroxybutyrate and almost completely abolished by a combination of the two. Regression analysis of the relationship between the total tumor concentration of lipolytic activity and the carcass fat obeys a multiplicative model (Fig. 6). This shows a large decrease in carcass fat with relatively low levels of lipolytic activity but that large amounts of activity are required for total depletion of body fat stores. Plasma from tumor-bearing animals has twice the lipolytic activity (1.29 ± 0.04 μmol FFA/h/ml of plasma) of control animals (0.63 ± 0.05 μmol FFA/h/ml of plasma) ($P < 0.001$), and from animals bearing the MAC13 tumor (0.596 μmol FFA/h/ml of plasma). This shows that the MAC16 tumor produces a circulatory lipolytic factor.

Cell-free extracts of the MAC16 tumor also caused an enhanced release of amino acids from mouse diaphragm, while extracts of the MAC13 and MAC15A tumors did not (Fig. 7). The proteolytic activity was completely abolished by both insulin and 8 mM sodium 3-hydroxybutyrate but not by 10 mM sodium chloride. Partial suppression was obtained with the trypsin inhibitor and complete suppression with 0.5 mM phenylmethylsulfonyl fluoride, heat, and acid. Neither RNase, DNase, nor ε-aminocaproic acid (10 mg/ml) had any effect on proteolytic activity. Regression analysis of the relationship between the total tumor proteolytic activity and the thigh and gastrocnemius muscle dry weights also obey a multiplicative model (Fig. 8).

Plasma from tumor-bearing animals had significant proteolytic activity (15.50 ± 4.41 nmol amino acids released/g diaphragm/2 h/ml plasma) while plasma from control animals had no proteolytic activity association with it ($P < 0.001$), and plasma from animals bearing the MAC13 tumor had low proteolytic activity (3.12 ± 2.91 nmol amino acids released/g diaphragm/2 h/ml plasma). This shows that the MAC16 tumor produces a circulatory proteolytic factor which may be responsible for some of the systemic effects.

DISCUSSION

The MAC16 is considered to be a good model of human cachexia, since host weight loss occurs with small tumor masses,

in analogy to the situation in humans where the tumor burden rarely exceeds 5% of the total body weight. In male mice weight loss begins when the MAC16 tumor exceeds 0.3% of the total body weight and reaches 30% when the tumor mass reaches 3% of the host body weight. In female mice host weight loss is more extensive, reaching 40% of the total body weight with a tumor burden of only 2.5%. There are very few experimental models of cachexia where weight loss occurs with such small tumor burdens. Strain *et al.* (14) have reported a human hypernephroma which produces greater than 25% weight loss in xenografted animals at tumor weights less than 5% of the total body weight, but most experimental tumors have to reach 20 to 40% of the total body weight before the symptoms of cachexia begin to appear. The tumor described in this report differs from that reported previously (11) in that weight loss occurs with much smaller tumor masses. This is due to the selection of an even more cachectic tumor during the transplantation.

Weight loss also occurs without a reduction in either caloric or fluid intake. Production of such an extensive weight loss with such a small tumor burden suggests that the tumor is not merely competing with the host for available nutrients. Also the total weight loss and the decrease of both body fat and skeletal muscle mass are directly proportional to the size of the tumor suggesting the production by the tumor of cachectic factors.

There is some evidence for the production of lipolytic substances by tumor cells, which appear to be independent of a host response. The evidence presented suggests a lipolytic factor produced by the MAC16 tumor, which is present in the circulation. Although as yet uncharacterized the lipolytic activity possibly differs from that described by Masuno *et al.* (7) which caused a suppression of food and water intake. The effects are also unlikely to be due to the production of cachectin (tumor necrosis factor) by tumor-associated macrophages, since there is no evidence of tumor necrosis factor elevation in the serum of mice bearing the MAC16 tumor or in MAC16 tumor extracts.[4]

The lipolytic substance appears to be specific for cachexia-inducing tumors, since two closely related colon adenocarcinomas, the MAC13 and MAC15A, neither of which produce cachexia *in vivo*, have a greatly reduced lipolytic activity when compared with the MAC16 tumor. The lipolytic factor is most probably a protein since it is nondialyzable, is destroyed by both heat and acid, and is not a prostaglandin since indomethacin has no effect on FFA release at concentrations up to 1 mM. Release of FFA is also not due to nonspecific proteolysis, since neither the trypsin inhibitor nor phenylmethylsulfonyl fluoride cause a significant reduction in lipolysis. Insulin is a very potent antilipolytic hormone and dramatically reduces the lipolytic activity of the MAC16 tumor extract. Ketone bodies are formed from excess FFA in the liver during starvation and high ketone body levels have been suggested to directly reduce lipolysis in adipose tissue (15). In the present experiments 3-hydroxybutyrate was shown to suppress the lipolytic activity of the MAC16 tumor and this effect was synergistic with insulin. The lipolysis produced by the tumor extract does respond to normal physiological stimuli. The presence of the lipid-mobilizing factor in the plasma of tumor-bearing mice suggests that lipolytic is not a local effect but that the tumor produced a circulatory factor to mobilize the host lipids, which may be required for tumor growth. The factor appears to be different from adrenaline, produced during fasting, since propranalol has no effect on lipid mobilization by the MAC16 tumor extract.

In addition to the loss of adipose tissue, animals given transplants of the MAC16 tumor show a decrease of skeletal muscle mass. Wasting of skeletal muscle has been attributed to depressed protein synthesis (16) but may also arise from an increased rate of catabolism. An increased proteolytic activity is often associated with the presence of malignant growth and has been implicated in the loss of growth control, invasiveness, and metastasis of tumors (17). Lazo (18) has suggested that the requirement by the tumor for essential amino acids, particularly leucine, leads to breakdown of the muscle stores. Although the MAC16 tumor does not show any evidence for metastasis, extracts of the tumor show the presence of proteolytic activity when measured by the rate of release of amino acids from mouse diaphragm as a model of skeletal muscle. The proteolytic activity appears to be confined to the MAC16 tumor since the two non-cachexia-inducing tumors, MAC13 and MAC15A, prevented rather than accelerated the release of amino acids from the mouse diaphragms. The amino acid release could not simply be attributed to leakage of materials from the intracellular pools since spontaneous release is inhibited by both insulin and 3-hydroxybutyrate and both insulin and 3-hydroxybutyrate completely inhibit the proteolytic activity of the MAC16 tumor fraction. The effect of sodium 3-hydroxybutyrate appears specific since sodium chloride has no effect on either spontaneous or induced release of amino acids from the diaphragms. Using a similar system Fulks *et al.* (19) showed that insulin stimulated protein synthesis and inhibited protein degradation while Palaioglogos and Felig (20) showed that ketone bodies decreased proteolysis in isolated rat diaphragms. The fact that insulin inhibits both the lipolytic and proteolytic activities of tumor homogenates may explain the ability of insulin to reverse experimental cancer cachexia (21). Phenylmethylsulfonyl fluoride, while having no effect on spontaneous hydrolysis, completely inhibited the induced release of amino acids by the MAC16 tumor extract, suggesting that the latter elaborates a serine protease. Part of the proteolytic activity can be attributed to a trypsin-like enzyme, since partial suppression of proteolytic activity was achieved with the trypsin inhibitor. A trypsin-like serine protease has also been shown to be produced by Walker 256 carcinoma cells (22). That the proteolytic activity is not due to a plasminogen activator is shown by the lack of inhibition by ε-aminocaproic acid. The presence of the proteolytic factor in the plasma of tumor-bearing mice suggests proteolysis is not a local effect but that the MAC16 tumor produces a circulatory proteolytic factor.

Thus the cachectic effect of the MAC16 tumor appears to arise from the production by the tumor of catabolic factors. Further characterization of these factors is in progress.

[4] S. Mahoney and M. J. Tisdale, unpublished results.

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Comparison of weight loss induced by recombinant tumour necrosis factor with that produced by a cachexia-inducing tumour

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Summary A comparison has been made of the cachectic effects produced by the transplantable murine adenocarcinoma of the mouse colon (MAC16) with tumour necrosis factor-α (cachectin). Tumour necrosis factor-α (TNF-α) produced a dose-related weight reduction that was accompanied by a decrease in both food and water intake. The degree of weight loss was directly proportional to the decreased food and water intake. In contrast weight loss produced by the MAC16 tumour occurred without a reduction in fluid or nutrient intake. Both the MAC16 tumour and TNF-α produced hypoglycaemia and a reduction in the circulatory level of free fatty acids (FFA), but had opposite effects on the level of plasma triglycerides with the MAC16 tumour-induced cachexia causing a decrease and TNF-α producing an increase. The MAC16 tumour elaborated a lipolytic factor which caused an immediate release of FFA from adipose tissue. In contrast TNF-α had no effect on mobilization of adipose triglycerides over a short time period. Both TNF-α and extracts from the MAC16 tumour caused an enhanced release of amino acids from mouse diaphragm, which was suppressible with indomethacin and heat labile. No TNF was detected in the MAC16 tumour or in the serum of tumour-bearing animals. Both tumour and non-tumour-bearing animals responded with a similar elevation of their serum TNF levels 90 min after a single injection of endotoxin. It is concluded that weight loss produced by TNF-α arises from an anorexic effect and that this differs from the complex metabolic changes associated with cancer cachexia.

We have been investigating a chemically induced, transplantable adenocarcinoma of the colon (MAC16), passaged in inbred NMRI mice as an experimental model of cachexia (Bibby *et al.*, 1987). This tumour produces weight loss at small tumour burdens (<1% of the host weight) and without a reduction in the intake of either food or water. The weight loss, which is directly proportional to the tumour weight, is associated with a decrease in both carcass fat and muscle dry weight (Beck & Tisdale, 1987). The cachectic effect of the tumour has been attributed to the production of both lipolytic and proteolytic factors, which are present in the circulation of tumour-bearing animals.

Endotoxin-induced cells of the reticuloendothelial system have been shown to elaborate a mediator called cachectin (tumour necrosis factor, TNF), which induces a state of cachexia in recipient animals (Cerami *et al.*, 1985). When chronically secreted by host macrophages cachectin has been suggested to contribute to a catabolic state, which ultimately leads to cachexia (Beutler & Cerami, 1986). Torti *et al.* (1985) have shown that cachectin acts to suppress the biosynthesis of several adipocyte-specific mRNA molecules and prevents morphological differentiation of pre-adipocytes. Lipoprotein lipase is one of the many enzymes whose transcription is suppressed by the action of this hormone (Price *et al.*, 1986*b*). Inhibition of lipoprotein lipase would prevent adipocytes from extracting fatty acids from plasma lipoproteins for storage. This would result in a net flux of lipid into the circulation, where the host defence could use it as an energy source. With chronic infectious challenge, however, wasting could persist and death would ensue (Beutler & Cerami, 1986).

In order to evaluate the role of TNF in cachexia we have compared the parameters contributing to weight loss in animals bearing the MAC16 tumour with that produced by human recombinant TNF-α, and sought to determine the presence of TNF either in tumour extracts or in the serum of tumour-bearing mice.

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Materials and methods

Animals

Pure strain NMRI mice (age 6–8 weeks) were purchased from Banting and Kingman, Hull and fed *ad libitum* a rat and mouse breeding diet (Pilsbury's, Birmingham, UK). All animals were given free access to water and both food and water intake were monitored daily. Fragments of the MAC16 or MAC13 tumours (1 × 2 mm in size) were implanted into the flank by means of a trocar as described (Bibby *et al.*, 1987). Positive takes can only be identified 14 days after transplantation.

TNF

Human recombinant TNF-α (6×10^7 U mg^{-1}) was kindly donated by Boehringer IngelheimLtd., Bracknell, Berks, and was stored at 4°C. The endotoxin content was <0.125 EU ml^{-1} and there was no proteolytic contâmination. Fresh solutions of TNF-α were made up daily in 0.9% NaCl and 200 μl of the appropriate concentration of TNF-α was injected into the tail veins of female NMRI mice. Controls were injected with 200 μl 0.9% NaCl. Body weights and food and water intake were monitored daily. A second injection of TNF-α was given 24 h after the first injection. Blood was removed by cardiac punture from animals under anaesthesia 1 h after the final injection of TNF-α.

Metabolite determinations

Blood glucose was determined on whole blood with the use of the o-toluidine reagent kit (Sigma Chemical Co., Dorset, UK). Free fatty acid (FFA) levels were measured in plasma with a Wako NEFA C kit (Alpha laboratories). Plasma triglycerides were determined with a triglyceride diagnostic kit (Sigma Diagnostic, Dorset, UK).

Primed TNF production

Non-tumour-bearing and MAC16 and MAC13 tumour-bearing male NMRI mice were administered 1.25 mg kg^{-1} *E. coli* lipopolysaccharide (Sigma Chemical Co., Dorset, UK) into the tail veins and blood was removed 1.5 h later by cardiac punture from animals under anaesthesia. Blood was allowed to clot, centrifuged and the resulting serum was used for TNF determinations.

TNF assay

TNF was determined by an *in vitro* method similar to that previously described by Ruff and Gifford (1981). L929 cells were seeded at a concentration of 3×10^4 per well into 96-well flat-bottom microtitre trays (Nunc., Denmark) in 100 µl RPMI 1640 medium (Gibco Europe, Paisley, Scotland) containing 10% foetal calf serum, and incubated at 37°C overnight under an atmosphere of 5% CO_2 in air. The medium was then removed and was replaced with varying dilutions of TNF-containing medium and actinomycin D (1 µg ml^{-1}) to a final volume of 100 µl. Controls contained only medium and actinomycin D. Internal standards contained medium with 1 unit of recombinant human TNF and actinomycin D. The plates were re-incubated for 16 to 18 h and the cells were stained with crystal violet. Rinsed and dried plates were enumerated spectrophotometrically at 570 nm on a Titerteck Multiscanner (Flow Laboratories) and the percentage of cell cytotoxicity was calculated as described by Flick and Gifford (1984, 1986).

Determination of lipolytic activity

The epididymal adipose tissue was removed from male BALB/c mice and minced in Krebs-Ringer bicarbonate buffer, pH 7.6. Approximately 50–100 mg of the adipose tissue was incubated with either the MAC16 tumour supernatant or TNF in a total volume of 0.25 ml of the Krebs-Ringer buffer. Controls containing adipose tissue and buffer alone were included in each experiment and the spontaneous release of free fatty acids (FFA) was subtracted from the values obtained with tumour present. The release of FFA by MAC16 tumour extracts was linear up to 2 h (Beck & Tisdale, 1987), and incubations were normally conducted for a 2 h period at 37°C. The concentration of FFA in the cell-free supernatants was determined immediately using a Wako NEFA C kit.

Determination of proteolytic activity

Male BALB/c mice were killed by cervical dislocation and diaphragms were carefully dissected out, blotted, cut in half, weighed and each half placed in a stoppered vial containing 0.75 ml Krebs-Ringer bicarbonate buffer and gassed for 20 sec with 5% CO_2 in air. Preincubations were carried out for 30 min at 37°C, and the diaphragms were then blotted and transferred to clean vials containing either tumour extract or TNF and the Krebs-Ringer buffer, in a total volume of 0.75 ml. The vials were gassed and incubated for a further 2 h at 37°C. Incubations were terminated by mixing 0.5 ml assay mixture with 0.125 ml of cold 50% TCA, mixing and centrifuging for 10 min at 3000 rpm. The supernatants were neutralised with 1 N NaOH and 0.2 ml of the neutralised sample was mixed with 1 ml of ninhydrin reagent, held in a boiling water bath for 20 min, and after dilution to 5 ml with *n*-propanol:water (1:1), the concentration of amino acids was determined spectrophotometrically at 570 nm. The spontaneous release of amino acids from the diaphragms in the absence of any additions was subtracted from the final readings.

Results

The characteristics of weight loss produced by the MAC16 adenocarcinoma passaged in NMRI mice has previously been reported (Bibby *et al.*, 1987, Beck and Tisdale, 1987). Briefly weight loss starts to occur when the tumour mass exceeds 0.1 g and reaches 10 g in a 30 g male mouse when the tumour mass is 0.7 g, representing just 2% of the weight of the animal. Both muscle and adipose mass decrease in direct proportion to the weight of the tumour (Beck & Tisdale, 1987). The average food intake in MAC16 tumour-bearing animals (15.1 ± 0.6 kcal day^{-1}) is not significantly different from that in non-tumour-bearing animals (14.9 ± 0.9 kcal day^{-1}). Also the water intake in tumour-bearing animals (4.6 ± 0.27 ml day^{-1}) does not differ from that of controls (4.8 ± 0.16 ml day^{-1}).

We have used female NMRI mice to study weight loss induced by TNF-α since they display a less aggressive behaviour than males, which may result in selective individuals being deprived food and water. Human recombinant TNF-α administered i.v. causes a dose-related weight loss after two separate injections over a 24 h period (Figure 1), which is significantly greater than saline injected controls at all concentrations of TNF-α employed. Qualitatively similar results were obtained with murine recombinant TNF-α, obtained from Dr W. Fiers, Biogent, Belgium (Marmenout *et al.*, 1985). No mortality was observed with any of the concentrations of TNF-α. This weight loss differs from that observed in MAC16 tumour-bearing animals in that it is associated with a dose-dependent decrease in both food (Figure 2) and water (Figure 3) consumption. The decrease in food and water intake is directly proportional to the decrease in body weight (Figure 4).

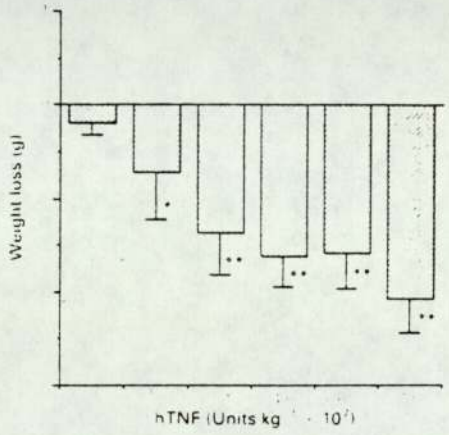

Figure 1 Effect of acute administration of TNF-α on the weight of female NMRI mice. Human recombinant TNF-α was administered i.v. as two separate injections over a 24 h period and the animals were killed 1 h after the last injection. The values represent the means ± s.e.m. for 4 to 11 animals for each concentration of TNF. $^*P<0.01$, $^{**}P<0.001$ from control by Students *t* test.

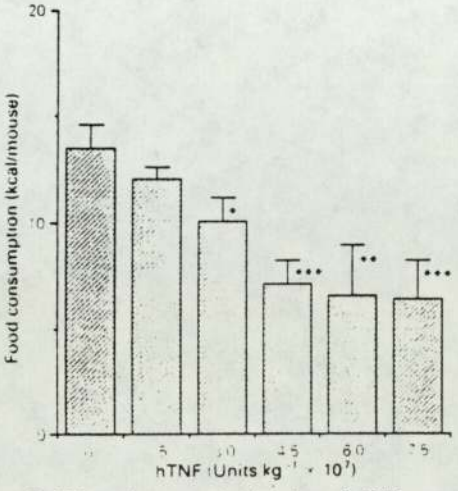

Figure 2 Effect of acute administration of TNF-α on food consumption of female NMRI mice during a 24 h period. The values represent the means ± s.e.m. for 4 to 11 animals for each concentration of TNF-α. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ from control by Students *t* test.

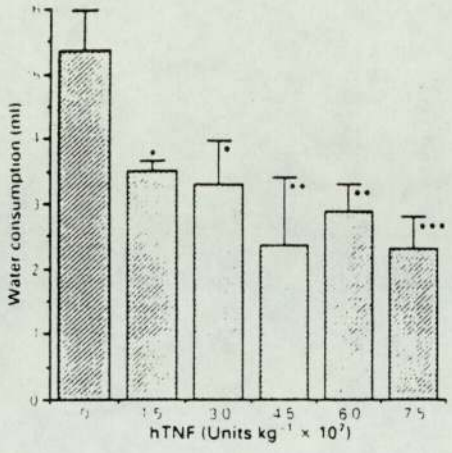

Figure 3 Effect of acute administration of TNF-α on water consumption of female NMRI mice during a 24 h period. The values represent the means ± s.e.m. for 4 to 11 animals for each concentration of TNF-α. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ from control by Students *t* test.

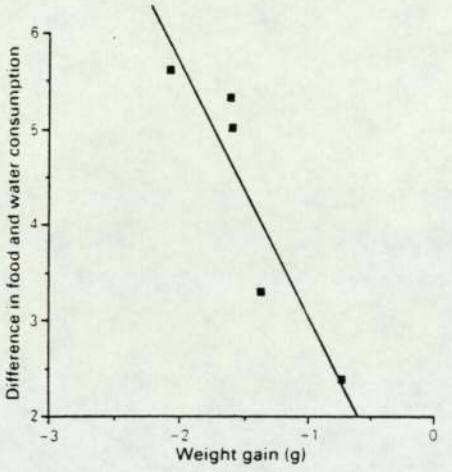

Figure 4 Variation of weight loss during a 24 h period after administration of TNF-α with the difference in food (kcal/mouse) and water (ml) consumption between a saline infused group and the TNF-α treated groups. The results were fitted to a linear model by means of a least squares analysis ($r=-0.99$).

Animals bearing the MAC16 tumour display a reduced blood glucose level. TNF-α treated mice also show a highly significant dose-related hypoglycaemia, which is much more pronounced than observed in weight-losing tumour-bearing animals (Table I). Plasma triglyceride levels are also reduced in tumour-bearing animals, whereas TNF-α causes an increase in circulatory triglycerides, presumably due to an inhibition of adipocyte lipoprotein lipase activity (Table I). Plasma levels of FFA are reduced after TNF-α administration, as might be expected from an inhibition of lipoprotein lipase and also in tumour-bearing animals, possibly due to increased tumour utilization.

The loss of body fat in MAC16 tumour-bearing animals has been correlated with the presence of a lipolytic substance produced by the tumour (Beck and Tisdale, 1987). This material is quantitated by the extent of release of FFA from mouse epididymal adipocytes. The results in Table II show that while extracts of the MAC16 tumour cause an enhanced release of FFA, TNF-α has no effect on the release of FFA under the conditions of the assay up to a concentration of 4×10^{5} units ml^{-1}. The MAC16 tumour also has high levels of proteolytic activity, which may be responsible for the muscle wasting (Beck & Tisdale, 1987) (Figure 5). Using the mouse diaphragm as a model of skeletal muscle, TNF-α at high concentrations also causes an enhanced release of amino acids (Figure 5). This effect is not due to contamination by endotoxin, since when the TNF-α is heated to 70°C for 15 min, which should destroy the TNF, but does not affect endotoxin, the proteolytic activity is completely destroyed. The proteolytic effect of TNF-α is almost completely suppressed by indomethacin and human α-1 antitrypsin. The proteolytic activity of the MAC16 tumour extract is also partially suppressed by indomethacin and there is a synergistic inhibition by a combination of indomethacin and antitrypsin (Figure 5). Proteolysis by trypsin is also inhibited by indomethacin. An enhanced amino acid release is also observed when diaphragms are incubated in the presence of PGE_2 or PGE_1, but not in the presence of $PGF_{1\alpha}$ or $PGF_{2\alpha}$ (Table III).

No TNF was detected either in the MAC16 tumour or in the serum of tumour-bearing mice using the L929 cytotoxicity assay. TNF was detected in the serum of non-tumour-bearing animals and in the serum of animals bearing the MAC16 and the non-cachexia inducing colon adenocarcinoma, MAC13, 90 min after a single i.v. injection of 25 μg endotoxin (Figure 6). However, there was no difference in the extent of response between non-tumour-bearing animals and animals bearing either type of tumour or in the levels of TNF in the two tumour types.

Discussion

The MAC16 tumour can be considered as an appropriate model for human cancer where weight loss occurs due to the biochemical effect of the tumour in patients with adequate

Table I Effect of recombinant TNF-α and the MAC16 tumour on the plasma level of glucose, FFA and triglycerides[a]

Treatment	*Glucose* (*mg 100 ml⁻¹*)	*FFA* (*mg 100 ml⁻¹*)	*Triglyceride* (*mM*)
Non-tumour-bearing	136 ± 5	29 ± 2	1.15 ± 0.11
Non-tumour-bearing (saline)	124 ± 5	32 ± 5	0.93 ± 0.31
MAC16 tumour-bearing	108 ± 11[b]	10 + 1[c]	0.50 ± 0.07[d]
TNF-α 0.25 mg kg⁻¹	82 ± 8[b]	17 ± 2[b]	2.72 ± 0.15[c]
TNF-α 0.5 mg kg⁻¹	74 ± 7[d]	15 ± 3[b]	2.52 ± 0.14[c]
TNF-α 0.75 mg kg⁻¹	59 ± 4[d]	19 ± 3[b]	2.24 ± 0.32[c]

[a]Results are given as means ± s.e.m.; [b]$P<0.05$ from non-tumour-bearing animals; [c]$P<0.01$ from non-tumour-bearing saline infused animals; [d]$P<0.001$ from non-tumour-bearing saline infused animals; [e]$P<0.005$ from non-tumour-bearing saline infused animals.

Table II Effect of recombinant TNF-α and the MAC16 tumour on the release of FFA from adipocytes

Addition	*nmol FFA mg protein^{-1} h^{-1} ± s.e.m.*[a]
MAC16 tumour extract	148.1 ± 8.3[c]
4×10^3 units TNF-α[b]	0
4×10^4 units TNF-α[b]	0
4×10^5 units TNF-α[b]	0

[a]Results are expressed as means ± s.e.m., [b]TNF-α in units ml^{-1} of the assay mixture; [c]Mean of 11 determinations.

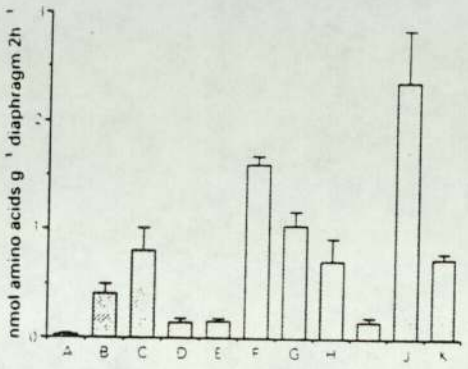

Figure 5 Rates of release of amino acids from mouse diaphragms by MAC16 tumour homogenate and TNF-α. (a) 10^3U TNF-α per assay. (b) 10^4U TNF-α per assay. (c) 10^5U TNF-α per assay. (d) 10^5U TNF-α + 1.0 mM indomethacin. (e) 10^5U TNF-α + 1 mg ml^{-1} α − 1 antitrypsin. (f) MAC16 tumour extract; 2.9 mg protein ml^{-1}. (g) MAC16 tumour extract + 1.0 mM indomethacin. (h) MAC16 tumour extract + 1 mg ml^{-1} antitrypsin. (i) MAC16 tumour extract + 1.0 mM indomethacin + 1 mg ml^{-1} antitrypsin. (j) Trypsin; 0.1 mg ml^{-1}. (k) Trypsin 0.1 mg ml^{-1} + 1.0 mM indomethacin. (b) and (c) $P<0.05$ from Krebs Ringer buffer alone. (d) and (e) $P<0.05$ from (c). (c) $P<0.05$ from (f). (h) and (i) $P<0.001$ from (f), by Students *t* test.

Table III Effect of prostaglandins on the release of amino acids from mouse diaphragm

	Concentration ($\mu g\,ml^{-1}$)	*nmoles amino acid released g.diaphragm^{-1} 2h^{-1} ± s.e.m.*
PGE_1	5	0.028 ± 0.024
	10	0.085 ± 0.006[a]
	20	0.234 ± 0.066[b]
PGE_2	5	0.069 ± 0.022[b]
	10	0.242 ± 0.079[b]
	20	0.369 ± 0.036[a]
PGF_1	5	0.000
	10	0.000
	20	0.040
PGF_2	5	0.000
	10	0.000
	20	0.000

[a]$P<0.005$ from spontaneous release; [b]$P<0.05$ from spontaneous release.

nutrient intake and without intestinal malfunction. In contrast TNF induces a state of anorexia and the ensuing weight loss is directly proportional to the decrease in food and water intake. A similar effect has been observed in mice injected with dialyzed conditioned medium obtained from lipopolysaccharide-induced peritoneal macrophages (Cerami *et al.*, 1985). Although all the experiments have been performed with human TNF-α similar results were obtained

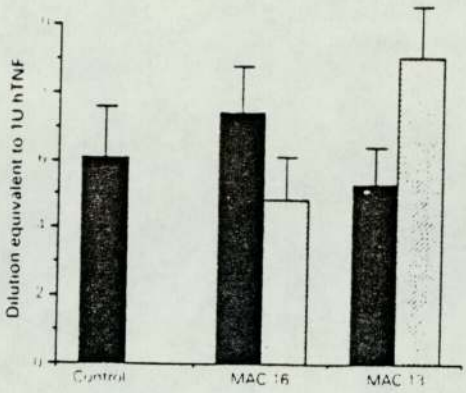

Figure 6 Production of TNF by endotoxin in unprimed mice. The TNF concentration in serum (■) and tumour (▨) was determined by means of the L929 cytotoxicity assay as described in **Materials and methods.**

with murine TNF-α. Marmenout *et al.* (1985) have shown that in spite of the apparent species specificity of TNF, human TNF is about 80% homologous to mouse TNF, and its hydrophilicity plot is also very similar.

The weight loss produced by both TNF-α and the MAC16 tumour is associated with hypoglycaemia, although TNF produces a more marked and possibly life-threatening decline in blood glucose levels. While administration of lipopolysaccharide has been shown to induce hypoglycaemia, Satomi *et al.* (1985) reported no hypoglycaemia in mice administered highly purified TNF. However, Kettlehut *et al.* (1987) have recently demonstrated large biphasic changes in blood glucose levels after TNF injection, with an initial hyperglycaemia followed by a sharp decrease in blood glucose. It has been suggested (Kettlehut *et al.*, 1987) that TNF may stimulate glucose uptake and oxidation contributing to the severe hypoglycaemia. In contrast the hypoglycaemia observed in animals bearing the MAC16 tumour probably arises from an increased consumption of glucose by the tumour (Tisdale & Brennan, 1986).

The MAC16 tumour and TNF-α differ as regards their effect on lipid metabolism in weight-losing animals. Thus, whereas animals bearing the MAC16 tumour have a reduced circulatory level of both FFA and triglycerides, TNF-α causes an increase in plasma triglyceride levels probably due to an inhibition of lipoprotein lipase activity. While lipoprotein lipase activity has been shown to be decreased in mice with the development of Sarcoma 180 (Masuno & Okuda, 1986) we have no evidence for an effect on lipoprotein lipase activity in animals bearing the MAC16 tumour, despite a massive loss of adipose tissue. This catabolism of adipose tissue has been attributed to the production by the tumour of a lipolytic factor (Beck & Tisdale, 1987). However, we have observed no increased breakdown of stored triglycerides in adipose tissue in the presence of TNF-α. While Kawakami *et al.* (1987) have reported that TNF-α increased the lipolysis of stored fat in 3T3-L1 adipocytes, even in the presence of 50 ng ml^{-1} of insulin, Price *et al.* (1986*b*) have shown that while crude preparations of TNF were able to suppress the activity of key lipogenic enzymes and stimulate lipolysis, recombinant TNF-α had no effect on either the ability of the adipocytes to synthesize and store or to mobilize triacylglycerols. The lipolytic activity of stimulated macrophages was attributed to interleukin 1, which both suppressed lipoprotein lipase activity and stimulated lipolysis (Price *et al.*, 1986*a*). Another possible reason for the absence of lipolysis we observed with our TNF-α preparation was the relatively short incubation time that we employed (2 h). Kawakami *et al.* (1987) did not observe an increase in glycerol production

in H111 cells until 12h after the addition of TNF-α, after which there was a linear increase in production up to 24h. Kettlehut *et al* (1987) have shown that the toxic and metabolic effects of TNF probably arise from an increased prostaglandin E_2 production since the cyclooxygenase inhibitors indomethacin or ibuprofen administered before TNF reduced the lethality and changes in blood glucose. We have shown (Beck & Tisdale, 1988) that the lipolytic substance elaborated by the MAC16 tumour is not a prostaglandin since indomethacin had no effect on FFA release at concentrations up to 1mM.

The MAC16 tumour also elaborates a serine-protease when measured by an accelerate rate of release of amino acids from mouse diaphragm as a model of skeletal muscle (Beck & Tisdale, 1987). Using a similar assay we have detected a proteolytic activity associated with high level of TNF-α. This activity was not due to the small amount of endotoxin contamination since it was destroyed by heating, and not due to the presence of endogenous proteases in the TNF-α preparation (Boehringer Ingelheim, pers. comm.). Proteolysis induced by both TNF-α and the MAC16 tumour extract is suppressible by indomethacin suggesting the possibility of a prostaglandin intermediate. We have shown that prostaglandins of the E series, but not of the F, are also effective in inducing amino acid release from mouse diaphgram. PGE_2 is believed to be an important stimulus for the production of intracellular proteases (Rodemann & Goldberg, 1982). Moreover, TNF-α has been reported to stimulate collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts (Dayer *et al*, 1985). This suggests that the enhanced release of amino acids from mouse diaphragm in the presence of TNF is due to an elevation of PGE_2 levels.

We have been unable to detect TNF either in the MAC16 tumour, or in the serum of tumour-bearing animals. Animals bearing either the MAC16 or the non-cachexing-inducing MAC13 colon adenocarcinomas do not respond to endotoxin with an increased TNF production compared with non-tumour bearing controls. This negates against a synergistic influence of the presence of a tumour on TNF production in response to endotoxin.

The results suggest that TNF has no role in the induction of cachexia seen in animals bearing the MAC16 tumour. Although we have compared the chronic secretion of factors produced by the MAC16 tumour with the acute effects of TNF we have shown (Mahony and Tisdale, unpublished results) that chronic exposure to TNF does not differ appreciably from the acute effects. Furthermore the weight loss produced by TNF appears to arise from an anorexic effect of this agent and this differs from the changes associated with cancer cachexia.

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