MECHANISMS AND TREATMENT OF WEIGHT LOSS IN CANCER

by

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DEDICATION

A.M.D.G.

To my mother
for her tireless
support, patience
and encouragement.

SUMMARY

MECHANISMS AND TREATMENT OF WEIGHT LOSS IN CANCER

Weight loss and the development of cancer cachexia are well known to contribute to the morbidity and mortality of cancer patients. Loss of body mass is most commonly due to an imbalance between energy intake and energy expenditure. The aim of this study was to examine the nature of the negative energy balance in cancer patients and evaluate several new approaches to redress the host-tumour imbalance in cancer cachexia.

Clearly an investigation of weight loss must first determine which compartments of the body are affected. Therefore, a study of the body composition of a group of cachectic lung cancer patients was undertaken. In this group of patients an 86% reduction of body fat and a 20% reduction of body protein was demonstrated. The loss of body protein was almost entirely due to severe depletion of skeletal muscle; non-muscle protein mass being preserved. Thus not only does cachexia lead to an overall reduction of body mass, but specific body compartments are affected by the disease.

These changes in body composition might, in part, be due to starvation. This hypothesis is supported by the observation that the food intake of the cachectic cancer patients was markedly reduced. However, it could not be determined whether a reduced food intake was present throughout the period of the patients' weight loss.

An alternative explanation for weight loss could be that the energy expenditure of cancer patients is increased. It has been proposed that an elevated energy expenditure in cancer patients might be due to the energy cost of an accelerated rate of whole body protein turnover. However, although patients with either lung or colon cancer had rates of whole body protein turnover which were 50 - 70% greater than weight-stable controls, this was not associated with an increased resting energy expenditure and was not necessarily associated with weight loss.

It was apparent that the relationship between energy expenditure, energy intake and weight loss might be better studied with an animal model of cancer cachexia. Total energy expenditure could be determined, food intake might be more accurately measured and interindividual variation reduced.

The mechanisms of weight loss were studied in two animal models of cancer cachexia. These were rats bearing the Walker 256 and mice bearing the MAC-16 tumour. The weight and protein loss of rats bearing the Walker tumour was shown to be equivalent to the weight and protein content of the tumour. In contrast, the tumour mass itself could not account for host weight loss in mice bearing the MAC-16 tumour.

When mice bearing the MAC-16 tumour had become cachectic weight loss was associated with a reduced food intake with no compensatory reduction in energy expenditure. However, the cause of the energy deficit during the early phase of weight loss could not be determined. As with studies on humans this might be explained by substantial interindividual variation, the errors of the methods available for the

measurement of food intake and energy expenditure and, the relatively small size of the changes in either variable necessary to account for host weight loss. The pattern of alterations in the food intake, energy expenditure and protein metabolism of mice bearing the MAC-16 tumour were markedly different from those observed in other murine models of cancer cachexia. This suggests that the mechanism of weight loss in cachexia may depend on the particular model studied.

Several methods of treating cachexia were evaluated. It has been proposed that many tumours are dependent on glucose as an energy source. In order to maintain a glucose supply to the tumour, host muscle protein is broken down to supply amino acids for an increased de novo synthesis of glucose in the liver. Ketone bodies are fat-derived energy substrates which may act to reduce gluconeogenesis by inhibiting muscle protein degradation. Thus the induction of systemic ketosis might be a method of selectively feeding the host whilst reducing the supply of glucose to the tumour. However, systemic ketosis had no effect on host nitrogen losses in either humans with cancer cachexia or rats bearing the Walker 256 tumour. Moreover, although blood glucose was slightly reduced in humans with cancer fed a ketogenic diet, this effect of the diet was not observed in rats bearing the Walker 256 tumour and there was no reduction in tumour growth rate. Taken together these results indicate that systemic ketosis is unlikely to improve the host-tumour imbalance of cachexia.

Clearly the best way to cure cachexia is to cure the cancer. Unfortunately, for the majority of solid epithelial malignancies systemic anticancer therapy is largely ineffective and may actually worsen the nutritional status of the host. In an attempt to develop a

non-toxic method of controlling tumour growth within the cachectic host, a novel approach based on potential differences between host and tumour energy metabolism was evaluated. Many tumours are known to be glucose dependent and have mitochondria which are reduced in number and abnormal in morphology and enzyme content. Thus, in tumour cells there may be abnormalities of both of the major sources of intracellular ATP. The simultaneous manipulation of glycolysis and oxidative phosphorylation by administration of the mitochondrial inhibitor Rhodamine 6G during a period of hypoglycaemia was shown to have a synergistic antitumour effect in rats bearing the Walker 256 tumour. The treatment was non-toxic and might well form the basis of future therapy in patients with disseminated malignancy and weight loss.

In conclusion, a reduced food intake is probably the main cause of continuing weight loss in the cachectic cancer host. The incidence and mechanisms of an increased energy expenditure remain uncertain and may be different for each group of cancer patients examined. Selective manipulation of tumour energy metabolism may be one method of inhibiting tumour growth, thereby allowing reversal of the cachectic state.

CHAPTER 1

Introduction to the Thesis

Starvation and the metabolism of the cachectic cancer host

The majority of patients with malignant disease lose weight (Nixon et al., 1980) and a proportion become emaciated to the point where they appear to die of starvation (Warren, 1932; Inagaki, Rodriguez and Bodey, 1974). This syndrome is known as cancer cachexia. The term cachexia is derived from the Greek words kakos meaning bad and hexis meaning condition. The patient with cachexia is characterised by severe weight loss, lethargy, anorexia, early satiety and anaemia (Calman, 1982). In addition, numerous studies have documented abnormalities of host metabolism which together indicate that cancer cachexia is not the same as simple starvation (Brennan, 1977).

In order to understand the problem of cancer cachexia it is necessary first to understand both the normal mechanism of energy balance in the body and the changes involved during adaptation to starvation.

The body requires energy and this is obtained either from food or from body fuel stores. During a prolonged period without food the energy and amino acids which are normally provided by the diet have to be obtained by the breakdown of body tissues and it is this process which leads to the emaciation characteristic of starvation. Fat is the major energy reserve in the body and loss of adipose tissue during starvation is well tolerated. However, there is no specific store of protein in the body and daily amino acids requirements have to be met by the breakdown of structural and functional proteins. Clearly vital

organs such as the heart need to be preserved for as long as possible. Therefore, during prolonged starvation the body first mobilizes protein from low priority tissues such as skeletal muscle. Thus, the tissues most affected by starvation are adipose tissue and skeletal muscle. However, the selectivity of wasting is not absolute and visceral protein is also lost.

One factor which contributes to protein loss in early starvation is the inability of the brain to use free fatty acids as substrates for energy production. Glucose is the preferred fuel and this is obtained by the release of amino acids from skeletal muscle which are then converted into glucose by the liver and kidney (Owen et al., 1969). Thus during early starvation the body excretes about 12g of nitrogen per day principally as a result of muscle proteolysis. If this process continued unabated the duration of survival would be limited due to a rapid loss of lean tissue. However, in response to starvation, ketone bodies are generated in the liver from free fatty acids and these replace glucose as the main substrates for cerebral energy metabolism (Robinson and Williamson, 1980). Thus the need for amino acids for gluconeogenesis is greatly reduced, the rate of muscle proteolysis is decreased, and the rate of nitrogen excretion in compensated starvation is only about 2g of nitrogen per day (Cahill, 1970).

This ordered pattern of metabolic adaptation would appear to be disrupted by the presence of the tumour. Although anorexia is often present in cancer patients, weight loss frequently exceeds that expected from a reduced food intake alone (Costa et al., 1981). In contrast with compensated starvation the rate of gluconeogenesis from alanine (Waterhouse, Jeanpetre and Keilson, 1979), lactate (Waterhouse, 1974; Holroyde, Gabuzda and Putnam, 1975) and glycerol (Lundholm et

al., 1981) has been shown to be increased in various groups of cancer patients. The mechanism of this accelerated gluconeogenesis is not clearly established. In 1930 Warburg observed that tumours metabolise glucose via glycolysis at a high rate, whilst in normal cells this pathway is usually reserved for anaerobic conditions. Subsequently, several studies demonstrated a high rate of glycolysis in tumours and a dependence on glucose as an energy source (Gold, 1966; Shapot and Blinov, 1974; Demetrakopoulos, Linn and Amos, 1982). Gold (1968) proposed that this high glucose demand by the tumour is an important factor in the development of cachexia. He suggested that body fat and protein reserves are depleted in order to provide substrates for gluconeogenesis. The glucose so produced is metabolised by the tumour which releases lactate into the blood stream. The lactate is then transported to the host's liver where it is converted back to glucose. The cycle from glucose to lactate and back to glucose is known as the Cori cycle and a 2 - 3 fold increase in the activity of this cycle has been reported in cachectic cancer patients (Waterhouse, 1974). Since the anaerobic breakdown of glucose to lactic acid yields only two molecules of ATP and the resynthesis of glucose from lactate requires six molecules of ATP this cycle is very wasteful of energy compared with the potential 36 molecules of ATP via the aerobic oxidation of glucose. However, since cachexia can develop in the presence of a very small tumour burden (Costa, 1977) and since some tumours that produce cachexia do not have increased rates of glycolysis (Weinhouse, 1982) the suggestion that cachexia is caused by the high glucose demand of the tumour does not seem generally applicable.

Protein metabolism in the tumour bearing host also appears to be different from that of uncomplicated starvation. Lundholm and coworkers (1976) demonstrated a decreased rate of protein synthesis and

an increased rate of protein degradation in muscle biopsies from cachectic cancer patients. This observation would account for a net protein mobilization. Moreover the reduced protein synthesis rate is consistant with the pattern observed in starvation (McNurlan, Pain and Garlick, 1980). However, in contrast to the overall depletion of muscle protein, a marked increase in liver mass accompanies the growth of several tumours (Stewart and Begg, 1953; Lundholm et al., 1979; Heymsfield and McManus, 1985). Unlike the changes observed in starvation, the liver seems to be protected from the net negative nitrogen balance in the tumour bearing host such that an increased rate of hepatic protein synthesis has been described (Lundholm et al., 1979; Emery, Lovell and Rennie, 1984). An increased rate of protein synthesis in the liver and a decreased rate in skeletal muscle together with the additional protein synthesis occuring in the tumour is thought to result in an apparently normal to increased rate of protein synthesis and turnover in the whole body (Jeevanadam et al., 1984; Eden et al., 1984). This is in sharp contrast to the decreased rates of whole body protein turnover and synthesis seen during uncomplicated starvation (Rennie et al., 1982).

Fat metabolism in the cancer bearing host is also abnormal. Fat is mobilized as the major energy reserve during starvation; the free fatty acids released are oxidized to provide energy and the glycerol backbone is converted to glucose via gluconeogenesis. Administration of glucose during starvation reduces both free fatty acid oxidation and gluconeogenesis and it is thought that this effect is mediated by insulin (Butcher, 1968). In contrast, free fatty acid oxidation continues in the presence of administered glucose in cachectic cancer patients (Waterhouse and Kemperman, 1971).

Although these observations support the suggestion that cancer cachexia may not be entirely consistant with semistarvation the question remains as to which metabolic abnormalities are significant and how do they induce weight loss?

Mechanisms of weight loss in cancer

In order to determine possible mechanisms of weight loss in cancer patients it is necessary to consider the factors which keep the body weight stable in normal individuals. When the energy lost as mechanical work or as heat is balanced by the energy obtained from food in the diet the body weight remains stable. Therefore, loss or gain of body mass is due to an imbalance between energy intake and energy expenditure. A number of studies have looked for an imbalance in this relationship in cachexia. Walsh and coworkers (1983) and Cohn and colleagues (1981b) both demonstrated a marked reduction of food intake in cancer patients who had become severely cachectic. However, in contrast, when cancer patients were studied during the early phase of weight loss a reduction in food intake was not observed (Costa et al., 1981).

Whether or not food intake is reduced, an increased energy expenditure might account for the rapid weight loss and other symptoms of cachexia in cancer patients. When an individual is at rest and is not performing any mechanical work energy expenditure is equal to the amount of heat transferred from the body to the atmosphere. This resting energy expenditure can be determined by measuring the amount of heat given off by the body and this technique is known as direct calorimetry. However, in clinical medicine direct calorimetry is rarely employed to determine heat loss or heat production; rather

indirect calorimetry is the preferred method. This method relies upon the direct relationship between the rate of release of energy from the body and the rate of oxygen consumption and carbon dioxide production.

Numerous investigators have used indirect calorimetry to determine whether the resting energy expenditure of patients with cancer is elevated. Lindmark and coworkers (1984) and McFie and colleagues (1982) both demonstrated that the resting energy expenditure of patients with disseminated malingancy is increased by 5 - 20% when compared with controls. In contrast, Hansell and coworkers (1986) showed that there was no difference between the resting energy expenditure of large groups of patients with either lung, colon or gastric cancer and that of non-cancer controls. In another study (Dempsy et al., 1984) it was shown that approximately one third of cancer patients were hypermetabolic, one third were normal and one third were hypometabolic. However, even a 'normal' rate of energy expenditure would be inappropriate in the semistarving cancer host since the normal adaptive response to a reduction in energy intake is a decrease in energy expenditure.

The mechanisms whereby the energy expenditure of some cancer patients is increased or at least fails to adapt to the semistarving state are unknown. One proposal is that energy dependent metabolic cycles such as protein turnover or the glucose-lactate cycle (Cori cycle) are increased. However, the relation between these abnormalities and the metabolic rate, increased or otherwise, is not clear. Although some investigators have found a correlation between an increased Cori cycle activity and weight loss (Holroyde et al., 1975), other workers have failed to do so (Kokal et al., 1983). Similarly, whole body protein turnover has been shown to be elevated in some

groups of malnourished cancer patients (Jeevanandam et al., 1984; Eden et al., 1984) but not in others (Glass et al., 1983). Thus, as yet it is unclear whether the weight loss of cancer patients is primarily due to a reduced food intake, an increased energy expenditure or a combination of the two. Moreover, the mechanism of any proposed elevation of energy expenditure is not established.

Clearly in order to discriminate between these possibilities it is first necessary to determine which of the body compartments are reduced in cancer cachexia. Thus one of the primary aims of this study was to characterise the body composition changes that occur during the development of cachexia. And, secondly, to determine whether weight loss is caused primarily by a reduced food intake. This study is described in Chapter 5.

The possibility that host energy expenditure is increased by the presence of the tumour causing an increased rate of protein turnover in the host is considered in a study described in Chapter 6. A similar study, based on a novel animal model of cancer cachexia is described in Chapter 7.

The reasons for abnormalities of protein, fat, carbohydrate and energy metabolism which have been documented in the cancer host are unknown. Indeed, it is not even clear if the observed changes form a consistent pattern such that all cases of cancer cachexia can be classed as a single syndrome. Cachexia can arise in humans when a tumour is less than 0.01% of host weight (Morrison, 1976) yet some patients with very large tumours show no sign of cachexia (Stein,

1978). Thus it is unlikely that the metabolic demands of the tumour constitute a significant drain on host metabolism. An alternative theory is that tumours produce small molecular weight peptides which are capable of altering the activity of various host enzymes. Because this occurs in a random manner, a state of metabolic chaos is produced (Theologides, 1977). However, although human tumours are known to be capable of both exocrine (Coombes, 1982) and autocrine (Sporn and Todaro, 1980) secretion of polypeptides, no tumour specific product has yet been shown to be responsbile for the metabolic defects which are thought to contribute to cachexia.

It has also been suggested that some of the metabolic abnormalities in cancer cachexia are similar to those observed in trauma and sepsis (Brennan, 1977). The loss of body fat and protein in patients with trauma or sepsis is thought to result from a combination of altered hormone profiles superimposed on semistarvation (Wilmore, 1983). Many of the metabolic events of trauma or sepsis (e.g. hyperglycaemia, increased nitrogen loss) can be reproduced by a combined infusion of glucagon, cortisol and adrenalin (Bessey et al., 1984). However, several aspects such as fever and production of acute phase proteins are not observed folowing such an infusion. The latter phenomena may be due to peptide lymphokines (e.g. Interleukin I and tumour necrosis factor) which are produced by inflammatory cells in response to injury and infection (Dinarello, 1985). Moreover, these mediators may enhance net triglyceride mobilization from adipose tissue (Beutler et al., 1985 a and b) and amino acid release from skeletal muscle (Baracos et al., 1983). Thus, the inflammatory response and the release of these mediators may be a key component in the events leading to body wasting associated with disease. In humans with cancer, tumour

necrosis, destruction of normal tissue and infection are just some of the factors independent of tumour type that could elicit such an inflammatory response. The hypothesis that the metabolic abnormalities observed in cancer cachexia are due to mediators of the inflammatory response is examined further in Chapter 6 with particular reference to protein turnover.

Treatment of cancer cachexia

Clearly, the best way to resolve the nutritional and metabolic abnormalities resulting from the growth of a tumour is the removal of the tumour (Calman, 1982). However, for the majority of solid epithelial malignancies, once the tumour has spread beyond the organ of origin, chemotherapy, radiotherapy or surgery will not effect a cure and may even worsen the nutritional deficit of the patient (Ohnuma and Holland, 1977; Kokal, 1985). Thus, whilst the search continues for more effective antineoplastic therapy, attention has been focused on supportive measures to reduce the morbidity and mortality which result from the nutritional problems associated with progressive tumour growth (Copeland, Daly and Dudrick, 1977). However, it is not known whether the nutritional state of cancer patients is an independent determinant of survival (Van Eys, 1982). Recent studies have suggested that when there is a good response to antineoplastic therapy the nutritional status of the patient will return to normal (McR. Russel et al., 1984). In contrast, with progressive tumour growth there is a partial block to the repletion of lean tissue (Nixon et al., 1981; Cohn et al., 1982) and nutritional support will neither lengthen survival nor improve the efficacy or tolerance of antineoplastic therapy (Brennan, 1981). This

lack of benefit from conventional nutritional support has emphasised the need for an understanding of the basic mechanisms of weight loss in in cancer patients. Moreover, the demand for alternative approaches to the treatment of cachexia has become all the more acute.

Selective feeding of the cancer host

Stimulation of tumour growth rate may be one explanation for the failure of nutritional support to improve the survival of wasted cancer patients. Hyperalimentation has been shown to increase tumour growth rates in a number of tumour bearing animals (Popp, Morrison and Brennan, 1981; Cameron, 1981), but the effects in humans are unclear (Brennan, 1981). A possible strategy to overcome this problem may be to refeed the wasted cancer patients with an energy substrate that could be used by the host but not by the tumour (Magee et al., 1979; Tisdale and Brennan, 1983). This approach is based on the fact that many tumours are dependent on glucose (Gold, 1966; Gullino, Grantham and Courtney, 1967) rather than fat or ketone bodies for energy production (Cederbaum and Rubin, 1976; Tisdale and Brennan, 1983). In contrast, most host tissues can use lipid substrate or glucose equally well for energy metabolism. The use of lipid substrates to control cachexia and tumour growth in an animal model of cachexia is discussed in Chapter 8. A similar study in cancer patients is described in Chapter 9. However, in the latter study the effect of systemic ketosis on tumour growth rate is not assessed.

Selective inhibition of the tumour

Current cancer chemotherapy is only curative for a small minority of cancer patients with disseminated disease (Frei, 1985). This systemic form of therapy involves the use of cytotoxic agents to inhibit cell division within the tumour. These agents are non-selective since they also inhibit cell division within the tissues of the host. Their use is based on the assumption that the rate of cell division within the tumour is greater than that of the majority of host tissues. Clearly this is not always a valid assumption and such treatment is often limited by host toxicity. Thus, cancer chemotherapy is often only partially effective and may, in fact, exacerbate the nutritional consequences of progressive tumour growth (Donaldson and Lenon, 1979; Kokal, 1985). An alternative approach would be to evolve a more selective therapy based on the differences in energy metabolism between normal and malignant cells. Several tumours have been shown to have an abnormally high rate of anaerobic glycolysis (Warburg, 1930) and to be dependent on glucose as a major energy substrate (Gold, 1965; Shapot and Blinov, 1974; Demetrakopoulos, 1978). Furthermore, many tumours are known to have few mitochondria which are abnormal in morphology and enzyme content (Pedersen, 1978). Since glycolysis and oxidative phosphorylation are the two major pathways of energy production within the cell, these observations suggest that there are major differences between the energy metabolism of normal and of tumour cells. Thus, chapter 10 describes an attempt to inhibit tumour growth by selective inhibition of tumour energy metabolism.

The principle aims of this study can be summarised thus:

1) To characterise the nature and rate of tissue loss in patients with cancer cachexia.

2) To establish whether the rate of whole body protein turnover is increased in patients with cancer, and if so, whether this is associated with an increased resting energy expenditure and hence with weight loss.

3) To establish a representative animal model of human cachexia and to use this model to determine whether there is a metabolic component to weight loss.

4) To investigate in an animal system whether a host specific energy substrate might be used to inhibit both the development of cachexia and the growth of the tumour.

5) To investigate the effect of a host specific energy source on substrate levels and protein metabolism in cachectic cancer patients.

6) To investigate the possible use of tumour energy metabolism as a novel target for systemic antineoplastic therapy.

Layout of thesis

Each chapter begins with a self contained introduction and therefore details of the rationale behind the various stages of the work are not discussed here.

In order to determine the rate of whole body protein turnover in patients with cancer it was necessary to have a method which was both reproducible and easily performed in the clinical situation. Two

methods were available. One involved the administration of an amino acid labelled with a stable isotope of nitrogen and the other an amino acid labelled with a stable isotope of carbon. A primed, constant, 24h infusion of ^{15}N-glycine was chosen because it was the cheaper of the two methods and involved less disturbance of the patients. This method is described in Chapter 2.

The influence of protein turnover on host energy expenditure was also studied. Energy expenditure can be measured either by direct or indirect calorimetry. Indirect calorimetry, was the method more easily adapted to the clinical situation and is described in detail in Chapter 3. Protein metabolism and energy expenditure were studied in greater detail in a new murine model of cancer cachexia and the methods which were used are described at the end of Chapters 2 and 3 respectively.

Clearly, destructive chemical composition analysis of humans is not feasible and therefore a method was sought which would allow accurate determination of body composition in vivo. The technique of neutron activation analysis and whole body gamma spectrometry was chosen since not only did this allow accurate determination of chemical body composition in vivo but also enabled the division of the lean body mass into protein, water and mineral compartments. The details of this method are described in Chapter 4.

The nature and quantity of tissue loss in a group of cachectic lung cancer patients are described in Chapter 5. Patients who had lost approximately 30 per cent of their pre-illness weight underwent neutron activation body composition analysis. The results from these patients were compared with those from healthy controls who were matched for the age, sex and pre-illness stable weight of the cancer patients.

One possible mechanism for loss of weight was investigated in patients with either lung or colon cancer (Chapter 6). It had been suggested that whole body protein turnover might be elevated in the cancer host and that the energy cost of this might lead to a rise in host energy expenditure and thus initiate or worsen a negative energy balance. This hypothesis was investigated by measuring whole body protein turnover and resting energy expenditure simultaneously in both cancer and non-cancer patients with or without weight loss.

The nature and cause of the negative energy balance of the weight-losing cancer host was further investigated in Chapter 7. Body composition analysis was used to determine the nature of tissue loss in mice bearing the MAC-16 adenocarcinoma. Furthermore, food intake, energy expenditure and rates of protein synthesis were determined in the tumour bearing mice.

In addition to these studies of the mechanisms of weight loss in the cancer host, two new methods of treating the host tumour imbalance of cancer cachexia were evaluated. In the first, the use of systemic ketosis as a method of selectively feeding the host whilst reducing tumour glucose supply was appraised. The effect of a ketogenic diet on tumour growth rate and the development of host wasting was determined in rats bearing the Walker 256 tumour (Chapter 8). In Chapter 9 the effects of systemic ketosis on host nitrogen metabolism and blood susbstrate concentrations were determined in a group of cachectic cancer patients. However, the effect of ketosis on tumour growth rate was not determined in the latter study.

A more direct attempt to inhibit tumour growth rate is described in Chapter 10. This study involved the use of tumour energy metabolism

as a novel target for systemic anticancer therapy. Rats bearing the Walker 256 tumour were rendered hypoglycaemic by inhibition of gluconeogensis and at the same time an inhibitor of oxidative phosphorylation (Rhodamide 6G) was administered systemically. The effect on tumour growth rate of this simultaneous manipulation of both glycolysis and oxidative phosphorylation was then determined.

The results of each of these individual studies and the conclusions which can be reached with respect to the mechanisms and treatment of cancer cachexia are discussed in Chapter 11.

Measurement of protein synthesis and turnover in vivo

Introduction

Proteins play a crucial role in virtually all biological processes (Stryer, 1975) and are continually broken down and resynthesised within the body (Golden, Waterlow and Picou, 1977). Protein synthesis is a process which requires energy and some energy may be used in the hydrolysis of peptide bonds when protein is broken down (Waterlow, Garlick and Millward, 1978). A method was required to measure the rate of protein turnover in cancer patients and to determine the effect of such turnover on energy expenditure and energy balance. This chapter describes the principal features of the model used to study whole body protein turnover, synthesis and degradation in vivo. The two techniques that can be used to measure whole body protein turnover, and the reasons for selection of a primed, continuous 24h infusion of ^{15}N-glycine as the method of choice are discussed. The latter method is then detailed along with an assessment of its reproducibility in a young male volunteer. The technique used to measure protein synthesis rates in the tissues of a murine model of cancer cachexia is also outlined.

Model used to evaluate whole body protein turnover in vivo

The concept of a measurement which represents protein turnover in the whole body comes from the techniques which have been used to investigate protein metabolism in man. These techniques can be divided into precursor and end product methods. Both rely upon a basic two pool

model (Figure 2.1) where protein turnover is estimated from the flux of labelled amino acids into protein and into excretory pathways (Shipley and Clark, 1972). The label enters a single homogeneous free amino acid pool (the metabolic pool) from which it can either be oxidized, giving rise to CO_2 and urinary N, or it can be incorporated into protein. The protein of the body is regarded as a single pool which is continually recycling amino acids back into the metabolic pool, but because of its large size, acts as a sink for label entering it. Therefore, recycling of label is assumed not to occur. Thus, under steady state conditions, when the free amino acid pool is a constant size, the flux (Q) into the metabolic pool (from food intake, (I) and protein breakdown, (B) must equal the flux out of the metabolic pool (via protein synthesis, (S), and amino acid oxidation, (E). Thus:

$$Q = S + E = I + B.$$

It is therefore possible to calculate rates of whole body protein synthesis (S) and breakdown (B) by measuring flux (Q) and establishing the rate of nitrogen intake (I) and excretion (E). The end-product method and precursor method (representative amino acid method) are the two techniques which can be used to estimate Q.

End-product method

This approach has been used mainly with ^{15}N-labelled glycine. It is assumed that the labelled glycine donates ^{15}N via transamination to other amino acids and thus the ^{15}N acts as a label for the total free amino nitrogen pool. Since the urinary end-products of nitrogen metabolism (urea and ammonia) are derived solely from the free amino N pool their labelling can be taken as representative of that pool.

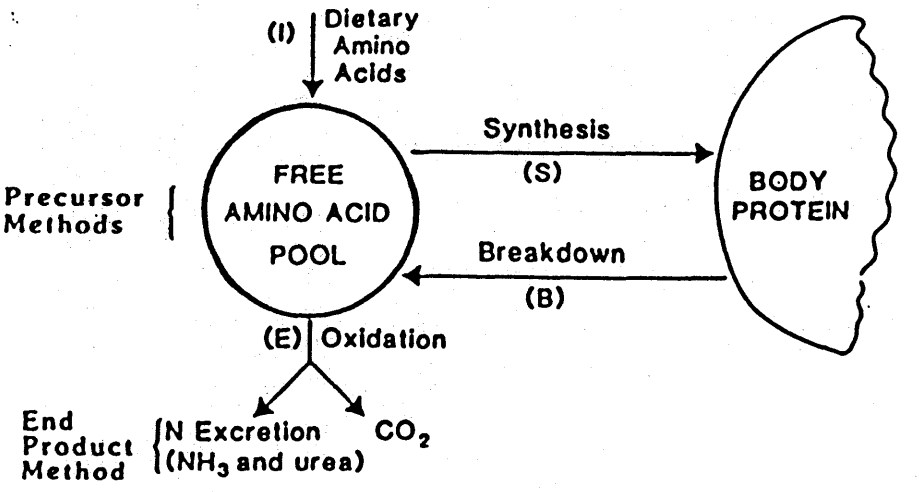


Figure 2.1 Two pool model of whole body protein metabolism. Flux of amino acids into the free pool comes from the diet (I) or from the breakdown of body proteins (B). Flux out of the free amino acid pool is via synthesis of body protein (S) or oxidation and excretion (E).

When a continuous infusion of ^{15}N-glycine is given at a rate d (mg isotope per hour) the specific activity (atom % excess ^{15}N) in the excreted end-product (urea or ammonia) reaches a plateau S max. It is assumed that in the steady state and at isotopic equilibrium the proportion of isotope excreted in the chosen end-product of nitrogen metabolism (ammonia or urea) is the same as the proportion of the nitrogen flux excreted in that same end product. This can be expressed:

$$\frac{em}{d} = \frac{Em}{Q}$$

where e_m = rate of excretion of ^{15}N in the chosen end product, E_m = rate of excretion of the end product, d = rate of infusion of ^{15}N and Q = the total nitrogen flux. Since in the steady state:

$$Smax = \frac{em}{Em} \Rightarrow \qquad Q = \frac{d}{Smax}$$

Thus, during a constant infusion of ^{15}N-glycine once plateau enrichment has been achieved, by measurment of the atom % excess of ^{15}N in urinary ammonia or urea, the rate of nitrogen flux in the whole body can be calculated (Waterlow, Garlick and Millward, 1978).

Precursor method

With this method measurement of whole body protein turnover is based on studying the kinetics of a single amino acid. A good example of this technique is when carboxyl-labelled ^{13}C or ^{14}C leucine is infused intravenously until the isotopic enrichment of the amino acid

in the plasma reaches a constant value (Waterlow, Garlick and Millward, 1978; Matthews et al., 1980). This plateau value can then be used to calculate the flux in a way analagous to that for ^{15}N-glycine. The rate of oxidation of the label can be calculated from the plateau isotopic enrichment of expired CO_2. A knowledge of the dietary intake of the amino acid then enables the rates of whole body protein synthesis and breakdown to be evaluated.

Rationale for selection of end-product method

The end product technique (^{15}N-glycine) was selected as the method of choice because:-

a) the method has been widely used (Picou and Taylor-Roberts, 1969; Fern et al., 1981; Jeevanandam et al., 1984), gives results similar to those obtained with ^{13}C or ^{14}C labelled leucine (Golden and Waterlow, 1977; Waterlow, Garlick and Millward 1978), and is sensitive to alterations in physiological states e.g. fasting (Rose et al., 1983).

b) By giving a primed, constant 24-hour infusion each patient study can be completed within 24 hours (Sim et al., 1980).

c) Sampling simply involves collection of urine and therefore disturbance to the patients and the need for nursing supervision is kept to a minimum.

d) The cost of the labelled amino acid (^{15}N-glycine: end product method) and the instrumentation required to measure the enrichment of ammonia and urea with ^{15}N (isotope ratio mass spectrometer) is several times less than the cost of [1 - ^{13}C] leucine (precursor method) and the gas chromatographic mass spectrometer

required to measure the isotopic enrichment of blood leucine or α-ketoisocaproate.

Details of end-product method

The protocol followed was that developed by Sim and coworkers (1980) and Sim, Ward and Johnson, (1984). After an overnight fast and during the 24h infusion the subject remained in bed and was allowed free access to water but not to food. The baseline enrichment of ammonia and urea was obtained from a urine sample taken at 08.00h. At 09.00h a bolus of 100mg ^{15}N-glycine was given and immediately followed by a continuous 24h infusion at the rate of 100mg ^{15}N-glycine/24h. The ^{15}N-glycine (99% atom percent ^{15}N : Amersham Int. Ltd., England) was dissolved in Na Cl (150mM) and sterilized by microfiltration. During the infusion, urine was collected in two consecutive 12 hour periods. A further sample was obtained at the end of the 24h infusion.

Urine collections were stored in the presence of 5ml of 6N HCl and 2mg of Chlorhexidine gluconate (ICI Ltd., Macclesfield, Cheshire, U.K.) in plastic containers. Urine samples were prepared for mass spectrometry according to the method of Read and coworkers (1982). The sodium/potassium form of the cationic ion exchange resin AG 50W - X8 (Biorad Laboratories Ltd., Watford, Hertfordshire, England) which specifically binds ammonia in neutral solution was used to extract ammonia from urine before and after treatment with urease (Type III, Sigma Ltd., Poole, Dorset, England). The resin ammonia complex was subsequently treated with alkaline hypobromite to liberate molecular nitrogen and ^{15}N-abundance measured with a double collector isotope ratio mass spectrometer (V.G. Micromass 602B, Cheshire, U.K.) with a

precision of 0.0008 atom % excess. Total urinary nitrogen was measured by the micro-Kjeldhl method (Fleck and Munro, 1965).

Rates of whole body protein turnover (Q) were calculated from the isotopic enrichment of ammonia and urea using the equation described on page 45. Rates of protein synthesis and degradation were derived from Q using the equation described on page 44. Since the subject was fasted, nitrogen intake was zero and therefore rates of protein turnover and degradation were the same.

Reproducibility of end-product method

Since it was possible to make only one estimate of protein turnover rates in cancer patients it was necessary to establish the reliability of this measurement. A small study was therefore carried out in which the protein turnover rate was estimated in a healthy volunteer on three separate occasions.

A primed, constant 24 hour infusion of ^{15}N-glycine was administered to a healthy male volunteer (65Kg : 25yrs) on three separate occasions. Rates of whole body protein turnover and synthesis were calculated from the isotopic enrichment of ammonia and urea obtained from a combined urine collection from the 12th - 24th hour of the infusion. The results are shown in Table 2.1.

The coefficient of variation of the rate of whole body protein turnover calculated from the isotopic enrichement of either ammonia or urea was less than 7%. The coefficient of variation of whole body protein synthesis with ammonia as the end product was 15.8% and with

INFUSION	TURNOVER (gProtein/Kg/d)		SYNTHESIS (gProtein/Kg/d)		URINARY NITROGEN (gN/day)
	Ammonia	Urea	Ammonia	Urea	
A	1.98	3.28	1.03	2.33	9.4
B	2.22	3.66	1.27	2.71	9.4
C	2.01	3.27	0.94	2.20	10.6
MEAN	2.07	3.4	1.08	2.41	9.8
C.V.*	6.3%	6.4%	15.8%	11.1%	7.1%

* Coefficient of variation

Table 2.1 Rates of whole body protein turnover and synthesis in a fasted male volunteer following a primed, constant 24h infusion of ^{15}N-glycine. Results are from 3 infusions given at weekly intervals.

urea was 11.1%. Since synthesis rates are calculated by subtracting the rate of nitrogen excretion from the rate of protein turnover (see page 44) the increased variability of the synthesis rates is probably accounted for by variation in daily urinary nitrogen excretion (Table 2.1). These results clearly indicate that the end-product method is a reproducible and reliable method for the determination of rates of whole body protein kinetics in vivo.

Validation of the protocol used to measure whole body protein turnover by the end-product method.

The calculation of protein turnover from the isotopic enrichment of urinary ammonia or urea during a continuous infusion of ^{15}N-glycine requires the achievement of a plateau of isotope excretion (see page 44). If an unprimed infusion of ^{15}N-glycine is administered intravenously, isotopic equilibrium in the urea pool can be achieved after about 48h (Picou and Taylor-Roberts, 1969). However, if at the beginning of constant infusion the relatively large urea pool is primed with a bolus of ^{15}N-glycine it has been suggested that isotopic equilibrium can be achieved within 12 - 18 h (Sim et al., 1980).

In order to confirm that plateau isotopic enrichment has been achieved, the rate of protein turnover is usually calculated from the mean isotope enrichment of several consecutive urine samples. However, a single urine collection over the second 12 hours of a 24h primed, constant infusion has been suggested as a method with which to avoid such multiple sampling (Sim, Ward and Johnson, 1984). This suggestion was confirmed by measuring the kinetics of isotope enrichment of ammonia and urea in a series of consecutive urine samples obtained from the same individual. This was done on three separate occasions. The results are shown in Table 2.2.

TIME	INFUSION A		INFUSION B		INFUSION C	
	(atom % excess)					
(h)*	Ammonia	Urea	Ammonia	Urea	Ammonia	Urea
0- 1	1.2529	0.0523	1.0003	0.0489	1.1121	0.0627
1- 2	0.2393	0.0367	0.2553	0.0388	0.2108	0.0442
2- 3	0.1468	0.0357	0.1504	0.0384	0.1407	0.0426
3- 4	0.1266	0.0427	0.1215	0.0298	0.1220	0.0447
4- 5	0.1005	0.0487	0.1216	0.0393	0.1078	0.0463
5- 6	0.0994	0.0483	0.1075	0.0408	0.1030	0.0457
6- 8	0.0911	0.0508	0.1062	0.0423	0.1047	0.0495
8-10	0.1169	0.0543	0.0880	0.0472	0.0898	0.0512
10-12	0.1177	0.0629	0.1045	0.0494	0.0868	0.0543
12-16	0.1157	0.0654	0.1018	0.0525	0.1064	0.0583
16-20	0.1046	0.0650	0.0898	0.0566	0.0992	0.0628
20-24	0.0939	0.0612	0.0764	0.0565	0.0932	0.0612
12-24	0.1003	0.0605	0.0894	0.0542	0.0990	0.0607
Mean**	0.1047	0.0639	0.0893	0.0552	0.0996	0.0668
CV**	10.4%	3.6%	14.2%	4.2%	6.6%	3.8%

* : time from start of ^{15}N-glycine infusion

** : mean and coefficient of variation of end product enrichment in urine collections taken from 12-16, 16-20 and 20-24 hours.

Table 2.2 Kinetics of ^{15}N enrichment of urinary ammonia and urea during a primed constant 24 hour infusion of ^{15}N-glycine. The subject was fasted for 12 hours prior to the study. Results are from 3 infusions given at weekly intervals.

Initially the isotope enrichment of urinary ammonia was very high but fell to a plateau within 4 hours of the start of the infusion. In contrast, the isotope enrichment of urea was only slightly elevated at first, fell to a trough level during the second hour of the infusion and then slowly increased to reach equilibrium which was maintained throughout the second twelve hours of the infusion. The coefficients of variation of isotope enrichment in ammonia and urea in the three urine samples from 12 to 24 hours were less than 15% and 5% respectively. Thus a single urine collection obtained over the second 12 hours of a 24 hour primed infusion of ^{15}N-glycine appears to be a valid and reproducible method of assessing plateau ^{15}N enrichment in both ammonia and urea.

Estimation of tissue specific rates of protein synthesis in mice.

The method used to measure rates of protein synthesis in the tissues of cachectic tumour bearing mice was that developed by Garlick, McNurlan and Preedy (1980). Protein synthesis rates are estimated by determining the rate of incorporation of labelled phenylalanine into protein. All possible amino acid precursor pools for protein synthesis are flooded by intravenous administration of a very large dose of radio labelled amino acid such that all pools reach the same specific activity which is maintained at an almost constant level during the period of measurement. This enables an accurate rate of protein synthesis to be calculated from measurements of the specific radioactivity of free and protein bound phenylalanine in the tissues.

Ten minutes after a tail vein injection of 1ml of 150μm phenylalanine and 1 μCi per gram of L - [4 - ^{3}H] phenylalanine the animals were lightly anaesthetised with diethyl ether and the liver,

kidney, heart and skeletal muscle were excised, immediately frozen in liquid nitrogen and ground to a powder. Thereafter the free phenylalanine was extracted into ice cold perchloric acid and the bound phenylalanine released by hydrolysing the protein with 6M HCl in a vacuum at 110°C for 24 hours.

Determination of the specific radioactivity of [^{3}H] phenylalanine involved its enzymatic conversion into B-phenylethylamine. This procedure was necessary because other amino acids (particularly tyrosine) became labelled following an injection of [^{3}H] phenylalanine. Phenylethylamine was extracted into an organic solvent and then into a dilute acid solution. An aliquot was removed for liquid scintillation counting and a further sample taken for estimation of the concentration of phenylethylamine. The measurement of phenylethylamine was based on the fluorescence enhancement of the amine with ninhydrin in the presence of the dipeptide leucylalanine. The fractional rate of protein synthesis (k_s) was then calculated from the specific radioactivity of phenylalanine in protein (S_B) at 10 minutes and the mean specific radioactivity of free phenylalanine in the tissue (S_A) between 0 and 10 minutes. The value of S_A was obtained by killing animals at 2 and 10 minutes and assuming a linear fall in specific activity between these two time points. The formula for calculating K_s was :

$$K_s = \frac{S_B \times 100}{S_A \times t}$$

where t is the incorporation time in days and the units of K_s are % per day.

Summary and Conclusions

A primed, constant, 24h infusion of ^{15}N-glycine is a reproducible method of estimating whole body protein turnover in humans. This method is used in Chapter 6 to determine the rate of protein turnover and its relation to energy expenditure in patients with lung or colon cancer. The method is also used in Chapter 9 to determine the effect of systemic ketosis on the protein metabolism of of patients with cancer cachexia.

The flooding dose of phenylalanine method is used in Chapter 7 to examine the protein metabolism of a new murine model of cancer cachexia.

CHAPTER 3

Measurement of energy expenditure in vivo

Introduction

In order to determine if weight loss in cancer cachexia is predominantly due to an increased energy expenditure rather than a decreased food intake, a method was required to measure energy expenditure in both humans and mice. There are two methods of measuring energy expenditure. Direct calorimetry relies upon measurement of the rate of heat exchange between the body and its environment. Indirect calorimetry estimates the rate of transformation of chemical energy into heat by oxidative metabolism. This is done by determining the rate of oxygen consumption and the rate of carbon dioxide production. Both methods rely upon the first law of thermodynamics which states that energy is neither lost nor gained when it is transferred from one form to another (eg. chemical energy to heat). Indirect calorimetry is the easier of the two methods to adapt for clinical investigation and gives results which agree with those obtained by direct calorimetry (Dauncy, 1980).

Measurement of resting energy expenditure in humans.

Resting energy expenditure was measured using an open circuit ventilated hood system housed in a temperature-controlled room. The subject's head was enclosed in a perspex canopy (Kinney et al., 1964) and the system made airtight by a flexible adhesive neck seal. The canopy was ventilated with air at a rate of 35 - 40 l/min; total flow

was measured by a wet gas meter (A. Wright Ltd., Tooting, London, U.K.). The gas mixing time constant of the head canopy and connecting piping was measured to be 36s, giving approximately a 95% response to a gas concentration change in 1 minute. Oxygen concentration difference across the canopy was measured by a dual-channel paramagnetic analyser (Servomex Ltd., Crowborough, Sussex, U.K.) and corrected to standard temperature and pressure. Carbon dioxide production was measured by an infra-red analyser (Sieger Ltd., Poole, Dorset, U.K.). The system provided measurements of $\dot{v}O_2$ and $\dot{v}CO_2$ for which the repeatability was $\pm$ 5% (95% confidence limits). The equipment was calibrated frequently using oxygen free nitrogen, 0.8% carbon dioxide (B.O.C. Ltd., Polmadie, Glasgow, U.K.) and air of known barometric pressure. The sensitivity and accuracy of the calorimeter was checked periodically by burning butane gas in the canopy. The 80 estimates of $\dot{v}O_2$ and $\dot{v}CO_2$ collected during each calorimetry run of 40 minutes were processed on line by a microprocessor and converted to mean energy production (Watts) and respiratory quotient (RQ) using the formulae of Wier (1949):

$$\text{REE (kcal/d)} = (3.9\ \dot{v}O_2 + 1.1\ \dot{v}CO_2)\ 1140 \text{ min/day}$$

where $\text{kcal/d} = \text{watts} \times 10.65$

$\dot{v}O_2$ = oxygen consumption (l/min)

$\dot{v}CO_2$ = carbon dioxide production (l/min)

$RQ = \dot{v}CO_2/\dot{v}O_2$

The patients had free access to water but not to food from 18.00h on the day prior to study. Each study began at 09.00h, patients having remained in bed since wakening. The 40 minute calorimeter run was

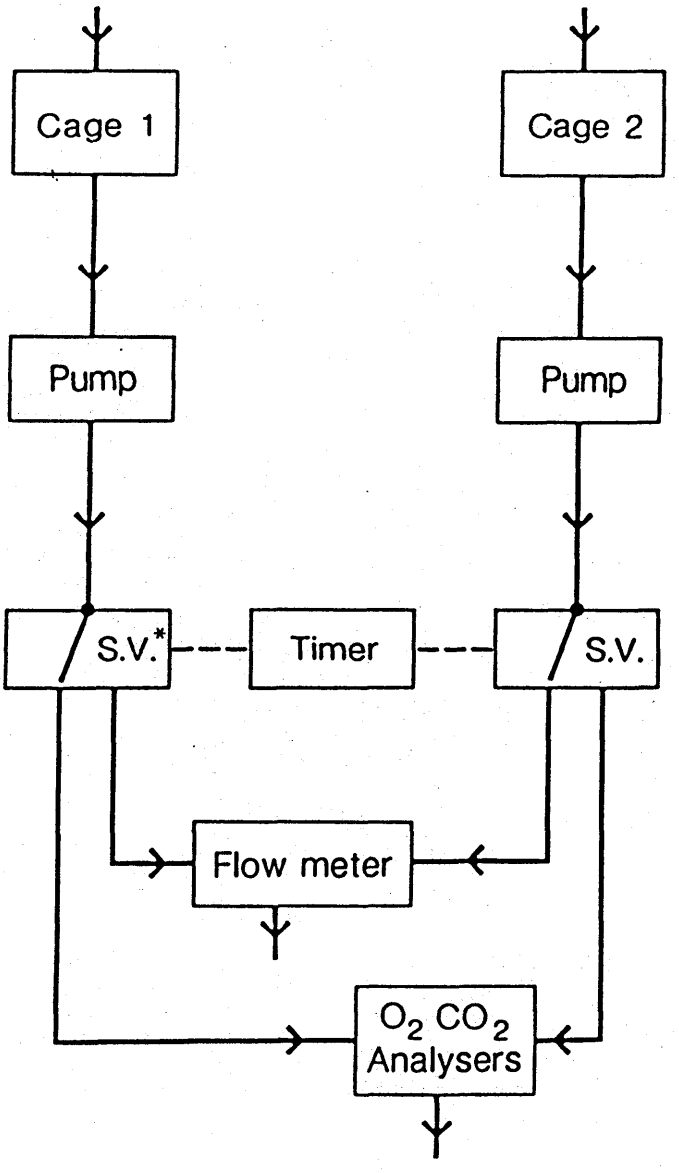
preceeded by a 30 minute acclimatization run.

Resting energy expenditure was expressed in relation to the patient's lean body mass. Lean body mass was derived from the measurement of total body water. Tritiated saline ($4MB_q$) was injected intravenously and serum samples were obtained 3 and 4 hours after injection. During the period of equilibrium all urine passed was collected to measure the loss of tritium in urine. Lean body mass was derived from the volume of body water assuming that lean tissue contains 73% water (Belcher and Vetter, 1971).

Measurement of daily energy expenditure of mice.

Levels of oxygen consumption and carbon dioxide production were obtained for each animal throughout the course of the investigation. Two animals were studied simultaneously using two glass metabolic cages (Minor Metabowl, H89/17/171, Jencons Scientific Ltd., Leighton Buzzard, Bedfordshire, England).

Ventilating room air was circulated through each cage at a small negative pressure (5mmHg) by pumps (Figure 3.1). Airflow was controlled and set within the range 275 to 325ml/min. The exhaust air from each cage was connected alternately to a wet gas meter (A. Wright Ltd., Tooting, London, U.K., England) for measurement of flow, then to gas analysers (Servomex OA150 Paramagnetic Analyser, LIRA Carbon Dioxide Analyser) to determine oxygen and carbon dioxide concentrations. This was accomplished by a timer which controlled two solenoid valves. Another solenoid valve (not shown in Figure 3.1) was



*S.V. = Solenoid Valve

Figure 3.1 Design of small animal indirect calorimeter. Room air is circulated through each metabolism cage by a pump. A solenoid valve directs the air either to the air flow meter or to the gas analysers. Each solenoid valve is controlled by a timer (not shown) which alternates the position of the valve every 5 minutes so that when the rate of flow of air through cage 1 is measured, the concentration of O_2 and CO_2 in the air from cage 2 is analysed.

energised for 5 minutes every 3 hours. This allowed room air to flow through reference and analysis cells of the oxygen analyser to measure baseline drift.

Prior to a run, both gas analysers were calibrated using oxygen free nitrogen as zero gas and a certificated reference stadard gas for the carbon dioxide analyser. Room air was used as the 'span' gas for the oxygen analyser.

As airflow was not measured when exhaust air was connected to the gas analysers, it was important that the resistance in the gas analysers' air circuit matched the resistance in the gas meter circuit. Thus at the beginning of each run, the air flow rate into each pump was measured with the solenoid valve in both positions and the two rates equated.

The wet gas meter has a multi-slotted disc from which electrical pulses proportional to flow rate can be obtained. A changeover relay controlled by the 5 minute timer was connected to the gas meter's electrical signal. Pulses from the gas meter were directed by this relay to one of two electromechanical counters so that the count accumulated by each counter was particular to a single channel. At the end of a run, the total count recorded by each counter was doubled and divided by the total run time to give the average flow rate.

In addition, an analogue signal of flow rate (derived from the gas meter's pulses) was recorded on one channel of a potentiometric

recorder. The other channels were connected to the oxygen and carbon dioxide analysers.

Average values for oxygen consumption and carbon dioxide production over the 24hour run were obtained by planimetry of the traces. Estimates of $\dot{v}O_2$ and $\dot{v}CO_2$ were converted to energy production using the formulae of Weir (1949).

Summary and Conclusions

Indirect calorimetry can be adapted for both clinical and animal studies. These methods are used in Chapters 6 and 7 to estimate the energy expenditure of patients and mice with and without cancer in order to determine whether energy expenditure is increased in cancer cachexia.

CHAPTER 4

Determination of body composition in vivo

Introduction

The syndrome of cancer cachexia is characterized by severe weight loss, anorexia, early satiety, lethargy and anaemia (Calman, 1982). However, these rather vague clinical descriptions provide little information concerning the alterations in nutritional status and the nature of tissue loss that occurs in patients with this condition. Body composition analysis is one of the most precise and accurate methods with which to determine nutritional status (Shizgal, 1985). Moreover, comparative measurements before and after weight loss allows the quantity and rate of tissue loss to be determined. This information can be used to assess the long term energy and nitrogen deficit which has been incurred by the patient.

In this chapter the various methods available to determine body composition in vivo are discussed, the rationale for the use of neutron activation body composition analysis and the basic principals concerning the technique are outlined. The model of body composition which was used to interpret the data provided by neutron activation analysis is also described.

Current methods used to determine body composition in vivo.

The body can be considered to consist of two compartments of relatively constant composition but which are distinctly different;

the compartments are 1) the body fat, and 2) the fat free mass or lean body mass. Since total body weight represents the sum of body fat plus lean body mass, measurement of either of these compartments can be used to estimate the other.

The body fat compartment is anhydrous, contains no potassium, and has a fairly constant density of about 0.90 x $10^3 Kg/m^3$. On the other hand, the lean body mass compartment has a density of about 1.10 x $10^3 Kg/m^3$ (Durnin and Womersley, 1974), a fairly constant potassium content (Boddy et al., 1972) and a water content of about 730g/Kg (Belcher and Vetter, 1971). Thus measurement of body density, total body potassium, or total body water allows a calculation of the relative proportion of fat and lean tissue in the normal individual. Body density can be measured directly by underwater weighing (Durnin and Rahaman, 1967) or indirectly by using the relationship between skinfold thickness and body density as documented by Durnin and Womersley (1974). Total body potassium can be analysed by measurement of the gamma irradiation emitted from the naturally occurring radioactive isotope of potassium, ^{40}K (Boddy et al., 1971). Total body water can be estimated by the standard tritiated water dilution technique as described by Vaughan and Bowling (1961). However, the accuracy of body composition analysis by these methods is limited by the variability of the composition and density of the lean body mass compartment in wasting disease (Moore et al., 1963).

Such variability is thought to arise because the lean body mass consists of two main components, namely, the cellular mass and the extracellular mass. In wasting disease it is recognised that the main

losses are from the cellular mass leaving a higher proportion of extracellular mass (James et al., 1984). Since tissues such as bone matrix make up the extracellular mass and have a low potassium and water content and also a high density this upsets the constancy of the lean body mass as a reference for density or compositional ratio.

Neutron activation body composition analysis.

One approach to the heterogeneity of the fat free mass is to measure directly the individual elements which make up the lean body mass. This can be done by application of the techniques of neutron activation and whole body gamma spectrometry (Cohn et al., 1981a). In this technique total body nitrogen, sodium, chloride, calcium and phosphorous can be determined by counting the induced radioactivity (gamma rays) resulting from the interaction between fast neutrons and body tissues. The particular nuclear reactions and the corresponding energies of the gamma irradiation emitted as a consequence of these reactions which are then used to calculate total body nitrogen, sodium, chloride, calcium and phosphorous are shown in Table 4.1. In addition, total body potassium is measured in the same apparatus via the natural radioactivity emitted by ^{40}K.

Details of neutron activation facility.

Patient and animal body composition studies were performed in the clinical facility at The Scottish Universities Research and Reactor

Element	Reaction	Gamma Ray Energy (MeV)	Yield %	Half life (min)
NITROGEN	$^{14}N(n,2n)^{13}N$	0.511	200	9.97
SODIUM	$^{23}Na(n,\gamma)^{24}Na$	1.37	100	901.8
CHLORINE	$^{37}Cl(n,\gamma)^{38}Cl$	2.75 1.6	100 38	37.18
CALCIUM	$^{48}Ca(n,\gamma)^{49}Ca$	2.17 3.1	47 89	8.72
PHOSPHOROUS	$^{31}P(n,\alpha)^{28}Al$	4.1 1.78	10 100	2.243

Table 4.1 Principal neutron activation reactions in vivo.

Centre, East Kilbride, Glasgow. At this centre induced and natural gamma irradiation is measured in a scanning bed counter (Boddy et al., 1975). This consists of two 29cm diameter x 10cm thick sodium iodide detectors mounted above and below the bed on which the patient lies. The apparatus is contained within a 10cm thick lead shadow shield. Bilateral scanning irradiation is conducted in an adjoining facility which uses two 14MeV sealed tube neutron generators which each operate at 10^{10} neutrons per second. The neutron generators are housed in a massive concrete shield. The patient lies on a couch which is driven through the tunnel and is irradiated during a single pass between the neutron generators (Williams et al., 1978).

Patient procedure for neutron activation analysis

Before irradiation the subject is passed through the shadow shield counter (30 scans taking about 20 minutes) in order to measure total body potassium and the total background count rate arising from the bodies' natural radioactivity. The subject is then transferred to the irradiation facility. An average dose equivalent of 10mSv (1 rem), principally due to fast neutrons but with about 5% due to gamma radiation is delivered at the body surface during the irradiation. A precise measurement of the neutron fluence to which the subject is exposed is obtained from the activation of four copper bars (160g each) attached to the edge of the irradiation bed. The induced ^{62}Cu activity is subsequently counted in a 7.5 x 7.5cm Sodium iodide Tl well counter.

After irradiation the subject is transferred back to the shadow shield whole body monitor and a further 30 scans are performed. This number of scans is used in order to obtain results with statistical counting errors of 3% or less for the average subject. Spectra obtained from the scans after irradiation are summed together in three groups: the first 5 scans; the next 10; and the last 15. The first 15 scans are made starting about 90s after the end of irradiation. The last 15 scans are begun 30 minutes after the end of irradiation since these are used to determine the longer lived products (^{38}Cl, ^{24}Na) after decay of the shorter lived ones. In total, each study lasts about 90 minutes.

The gamma spectra are corrected for Compton contributions, detector activation, annihilation radiation scans other than ^{13}N and interfering activities before computing the results. The precision of the method, represented by the average standard deviation of replicate phantom measurements, is $\pm$ 2.9% (Williams et al., 1978)

Small animals (eg. rats) can also undergo neutron activation body composition analysis *in vivo* with the facilities at the Reactor Centre, East Kilbride. The modifications necessary to the shadow shield body monitor, irradiation facility and experimental protocol are described by Preston and coworkers (1985).

Compartmental model used in analysis of NAA data

Neutron activation analysis and gamma spectrometry *in vivo* can provide a precise and accurate measurement of total body N, P, Na, Cl, Ca and K. From the measurement of these elements it is possible to determine total body protein, water and minerals. Total body protein (g) is derived by multiplying total body nitrogen (g) by 6.25. Extracellular water (ECW) is derived from total body chloride (TBCl), the major extracellular anion, using the formula : ECW (l) = 0.9 [TB Cl (mmol)/plasma Cl (mmol/l)] (Cohn, 1981c). Intracellular water (ICW) is derived from total body potassium (TBK), the major intracellular anion, using the formula: ICW(l) = TBK (mmol)/161 (McR. Russell et al., 1983). Finally, body mineral content is calculated from the sum of total body Na, K, Cl, Ca, P and a predicted value for mineral oxygen (I.C.R.P., 1975). Since lean body mass is the sum of body protein

water and minerals, body fat can then be calculated by subtracting lean body mass (Kg) from total body weight (Kg).

The body protein mass can be further divided into muscle and non muscle protein due to the different ratio of potassium to nitrogen in these two compartments. The concentration of potassium in relation to that of nitrogen in muscle is more than twice that in other lean tissues. Therefore a low proportion of muscle in the body will be reflected in a low ratio of total body potassium to total body nitrogen. Burkinshaw, Hill and Morgan (1978) summarized the mean values for the concentration of nitrogen and potassium in muscle and non-muscle lean tissue and derived the equations which were used to calculate the mass and protein contents of muscle and non-muscle lean tissues from total body nitrogen and potassium.

Summary and Conclusions

Neutron activation analysis and whole body gamma spectrometry in vivo can provide a precise and accurate measurement of total body N, P, Na, Cl, Ca and K. This non-destructive method of body composition analysis can be used in animals ranging from small rodents to man. These methods are used in Chapters 5 and 7 to estimate the nature of tissue loss in humans and rodents with cancer cachexia.

CHAPTER 5

Changes in body composition associated with cancer cachexia in humans

Introduction

Patients with cancer cachexia suffer from extensive body wasting and this is thought to contribute to patient morbidity and mortality (Warren, 1932; Inagaki, 1974). However, neither the cause, mechanism or nature of tissue loss in cachectic cancer patients is clearly established (Calman, 1982). One method of determining the nature of tissue loss in wasting disease is to measure changes in whole body composition. Until recently, indirect measurement of body composition was the chief source of such information (Moore et al, 1963). However, neutron activation and whole body gamma spectrometry can now provide direct measurements of body elements and thereby detailed quantification of body composition (Cohn et al, 1981c).

Previous body composition studies have generally examined cancer patients with a weight loss of only 5 - 15% (Warnold, Lundholm and Schersten, 1978; Burke, Bryson and Kark, 1980; Cohn et al, 1981b). However, this is not representative of the severe weight loss observed in cancer cachexia. Furthermore, these studies have examined heterogenous groups of patients with various types and stages of malignant disease many of whom have already undergone different forms of therapy. As discussed previously it is unlikely that cancer cachexia is a single phenomenon or that the mechanisms of weight loss are specific to a tumour type (Costa and Donaldson, 1979; see also Chapter 1). It is, therefore, improbable that the documented changes accurately characterise tumour specific alterations in body composition.

The aim of this study was to determine the alterations in body composition which occur in non-small-cell lung cancer patients with severe weight loss (> 25% loss of pre-illness stable weight). Patients with this type of tumour were chosen because of the high incidence of lung cancer in the general population and the fact that weight loss is the major predictive factor of poor survival in this disease (Lanzotti et al, 1977). Moreover, lung cancer does not primarily involve the gastrointestinal tract and many patients do not receive conventional therapy except for palliation of symptoms. Lung cancer patients, therefore, form an important, uniform group whose nutritional and metabolic status is primarily altered by direct tumour effects and not by gastrointestinal malfunction or antineoplastic therapy.

Probably the best method to study the effects of cancer cachexia on body composition would be to make sequential measurements on the same individuals throughout the natural course of their disease. Unfortunately, sequential measurements in cancer patients are difficult because it is impossible at present to predict who will lose weight and who will not. Moreover, the natural course of the disease is modified by the effects of surgery, chemotherapy or radiotherapy (Kokal, 1985), and patients are often lost to follow up due to a sudden deterioration in their condition. An alternative approach would be to compare the body composition of cancer patients who were already cachectic with that of weight stable controls who had been matched for the pre-illness weight of the cancer patients.
Unfortunately, many patients do not know their usual stable weight. Therefore, the main error in this approach is the validity of the patients pre-illness stable weight.

In this study the nature and extent of tissue loss in a group of six cachectic lung cancer patients was determined by comparing their body composition with that of a reference population who had been matched for the age, sex, height and pre-illness stable weight of the cancer patients. To ensure the validity of the cancer patient's pre-illness stable weight only patients who had been weight stable at their first clinic visit, had had their weight checked at that visit, and who had subsequently lost weight were included in the study. To improve the quality of the comparison, four control subjects were matched with each of the cancer patients. A six compartment model consisting of total body fat, muscle protein, non muscle protein, intracellular water, extracellular water and body minerals was constructed from measurements of total body nitrogen, sodium, chloride, calcium, phosphorous and potassium. These elements were measured by neutron activation and whole body gamma spectrometry.

Patients and methods

Cancer patients: The six cancer patients had histologicaly proven non-small-cell bronchial carcinoma. Disease staging included clinical examination, chest X-ray, full blood count and biochemical liver function tests. All patients had advanced metastatic disease (Stage III, WHO grading system). Two had small unilateral pleural effusions. None had overt ascites or ankle oedema. No patient had undergone surgery, radiotherapy or chemotherapy in the two months prior to study. The study was approved by the local hospital ethical committee. All patients gave written, informed consent.

The clinical details of the patients are shown in Table 5.1. There were 5 males and 1 female. Their mean age was 55 years. The mean weight of the cancer patients was 44.9 ± 3.4Kg (mean ± S.E.M.) which was 29% less than their pre-illness stable weight (63.3 ± 4.9Kg).

Control subjects: The 24 control subjects were selected from a group of over 1000 patients with mild to moderate hypertension. These individuals had undergone body composition analysis as part of a routine diagnostic protocol. Four control patients were matched for the age, sex, height and pre-illness stable weight of each of the cancer patients. The mean value of each group of 4 controls was then compared with that of each cancer patient.

The clinical details of the control patients are shown in Table 5.2. There was no significant difference between the age, sex, height and pre-illness stable weight of the controls compared with the cancer patients.

Nutritional and dietary assessment Serum albumin was measured on a commercial multichannel autoanalyser and urinary creatinine concentration was determined according to the modified method of Folin-Wu (Hawk, Oser and Summerson, 1947). Dietary protein and energy intakes were assessed by 24 hour dietary recall histories. At the time of body composition analysis patients were interviewed by an oncology dietician and their food intake noted for the previous 24 hours. The data was analysed by computer using a programme devised by Tayside Health Board which gave the protein, carbohydrate and fat contents of the diet. Desirable energy and protein intakes were obtained from standard tables (Documenta Giegy, Scientific Tables, 1962).

Patient	Sex	Age (yr)	Height (cm)	Weight (Kg)	Pre-illness Weight (Kg)	% Weight loss	Duration of Weight loss (M)
1	M	60	166	46.8	62.0	25	7
2	M	54	167	40.2	57.7	29	10
3	M	47	173	57.2	82.0	30	8
4	M	57	167	50.5	73.0	31	10
5	M	55	156	41.2	55.0	25	6
6	F	58	151	33.6	51.0	34	11
mean		55	163	44.9	63.3	29	9
S.E.M.		2	3	3.4	4.9	1	1

Table 5.1 Clinical details of cachectic lung cancer patients.

Subject	Sex	Age (Yr)	Height (cm)	Weight (Kg)
1	M	45	166	63.3
2	M	57	168	59.5
3	M	47	173	80.6
4	M	51	168	73.8
5	M	62	157	62.7
6	M	44	152	52.0
mean		51	164	65.3
S.E.M.		3	3	4.2

Table 5.2 Clinical details of weight stable controls.

Body composition analysis Total body sodium, nitrogen, chloride, calcium, phosphorous and potassium were determined by neutron activation and whole body gamma spectrometry as described in Chapter 4. Total body fat, muscle protein, non-muscle protein, extracellular water, intracellular water, and body minerals were derived as described on page 63.

Statistical analysis: Student's paired T-test was used to compare patient and control data.

Results

Nutritional state and dietary intakes: The mean serum albumin concentration of the lung cancer patients was 35% lower than that of the weight stable controls (28 $\pm$ 2 cf 43 $\pm$ 1g/l : $P < 0.01$, Table 5.3). Furthermore, daily urinary creatinine excretion was 69% lower in the lung cancer patients when compared with the controls (3.9 $\pm$ 0.3 cf 12.7 $\pm$ 1.3mmol/d; $P < 0.01$). The mean daily energy intake of the cancer patients was 5.0MJ/day which was 48% of their desirable intake (10.3MJ/day ; $P < 0.01$; Table 5.4). Their mean protein intake was 52g protein/day which was 75% of the desirable intake (75g protein/d : $P < 0.01$). The food intake of the control patients was not measured.

Body composition of cancer patients and controls: The total body nitrogen, sodium, chloride, calcium, phosphorous and potassium of the cancer patients and the controls are shown in Table 5.5. The mean total body nitrogen of the cancer patients was 20% less than that of the controls (1413 $\pm$ 82 cf 1737 $\pm$ 121gN : $P < 0.01$). Furthermore, total body potassium was reduced by 33% (81 $\pm$ 6 cf 121 $\pm$ 10gK : $P < 0.01$). There was no significant difference in total body sodium (78 $\pm$ 8

Patient	Serum Albumin (g/l)	24h Urinary Creatinine (mmol/d)	Control	Serum Albumin (g/l)	24h Urinary Creatinine (mmol/d)
1	27	3.8	1	41	11.8
2	33	3.5	2	42	11.4
3	28	3.4	3	44	13.5
4	29	4.1	4	44	17.5
5	29	5.3	5	44	13.7
6	20	3.0	6	43	8.3
Mean	28	3.9		43	12.7
S.E.M.	2	0.3		1.0	1.3

Table 5.3 Serum albumin and daily urinary creatinine of lung cancer patients and controls.

Patient	Energy (KJ)	% Predicted	Protein (g)	% Predicted
1	4930	47	49	70
2	4780	47	24	42
3	6440	49	69	99
4	5120	45	59	84
5	5670	57	53	75
6	3000	43	56	80
Mean	4990	48	52	75
S.E.M.	470	2	6	8

Table 5.4 Dietary intake of cancer patients.

	NITROGEN (g)		POTASSIUM (g)		SODIUM (g)		CHLORIDE (g)		CALCIUM (g)		PHOSPHOROUS (g)	
	P*	C**	P	C	P	C	P	C	P	C	P	C
1	1472	1746	80	121	66	71	53	56	871	977	485	542
2	1562	1775	82	121	83	81	65	62	889	1011	553	572
3	1642	2080	98	147	115	91	112	70	1114	1160	663	634
4	1437	1983	90	143	78	79	65	63	789	1059	508	589
5	1282	1580	83	110	62	73	55	57	711	1001	431	536
6	1085	1255	55	82	64	56	70	52	604	760	352	427
Mean	1413[a]	1737[b]	81[a]	121[b]	78	75	70	60	830[c]	995[d]	499	550
s.e.m.	82	121	6	10	8	5	9	3	71	54	43	29

a v b : $P < 0.01$, c v d : $P < 0.05$ *P = Patient ** C = Control

Table 5.5 Elemental body composition of cachectic lung cancer patients and healthy controls

cf 75 ± 5gNa : P > 0.1). Total body chloride was slightly but not significantly greater in the cancer patients when compared with the controls (70 ± 9 cf 60 ± 3 gCl : P > 0.1) Total body calcium was 17% less in the cancer patients compared with the controls (830 ± 71 cf 995 ± 54 gCa : P < 0.05). However, although total body phosphorous was 9% lower in the cancer patients, this difference was not statistically significant (499 ± 43 cf 550 ± 29gP : P > 0.1).

The mass (Kg) of total body fat, muscle protein, non-muscle protein, intracellular water, extracellular water and minerals derived from the chemical composition of the cancer patients and the controls are shown in Table 5.6 and Figure 5.1. The total body fat of the cancer patients was 80% less than that of the controls (3.1 ± 2.0 cf 17.3 ± 1.7Kg : P < 0.01). Furthermore, muscle protein was reduced by 76% (0.7 ± 0.3 cf 2.9 ± 0.3Kg : P < 0.01). There was no significant difference between the non muscle protein mass of the cancer patients and that of the controls (8.1 ± 0.5 cf 8.0 ± 0.4Kg : P > 0.1). Intracellular water was 30% lower in the cancer patients (12.9 ± 0.9 cf 19.2 ± 1.5Kg : P < 0.01) but there was no significant difference in the mass of extracellular water of the two groups (17.5 ± 1.9 cf 15.1 ± 0.7Kg : P > 0.1). The total mineral mass of the cancer patients was 13% less than that of the controls (2.6 ± 0.2 cf 3.0 ± 0.2Kg : P < 0.05). The lean body mass (body protein, water and mineral content) was also 13% lower in the cancer patients when compared with the controls (41.8 ± 3.1 cf 48.1 ± 3.0Kg : P < 0.01).

	FAT (Kg)		MUSCLE PROTEIN (Kg)		NON MUSCLE PROTEIN (Kg)		INTRACELLULAR WATER (Kg)		EXTRACELLULAR WATER (Kg)		MINERALS (Kg)		LEAN BODY MASS (Kg)	
	P*	C**	P	C	P	C	P	C	P	C	P	C	P	C
1	8.6	16.2	0.3	2.9	8.9	8.1	12.7	19.2	13.7	14.1	2.6	3.0	38.2	47.1
2	-2.7	10.6	0.1	2.6	9.7	8.4	13.0	19.2	17.4	15.6	2.8	3.1	42.9	48.9
3	1.4	23.1	1.2	3.7	9.0	9.3	15.6	23.3	26.4	17.6	3.5	3.5	55.8	57.4
4	8.5	19.8	1.4	3.8	7.6	8.6	14.2	22.7	16.2	15.8	2.6	3.2	42.0	54.0
5	4.0	18.1	1.5	2.6	6.5	7.3	13.1	17.4	13.8	14.4	2.2	3.0	34.8	36.2
6	-1.2	15.8	-0.2	1.6	6.9	6.2	8.7	13.1	17.4	13.0	1.9	2.3	34.8	36.2
Mean	3.1[a]	17.3[b]	0.7[a]	2.9[b]	8.1	8.0	12.9[a]	19.2[b]	17.5	15.1	2.6[c]	3.0[d]	41.8	48.1
s.e.m.	2.0	1.7	0.3	0.3	0.5	0.4	0.9	1.5	1.9	0.7	0.2	0.2	3.1	3.0

a v b : $P < 0.01$, c v d : $P < 0.02$. *P = Patient **C = Control

Table 5.6 Compartmental body composition of cachectic lung cancer patients and healthy controls.

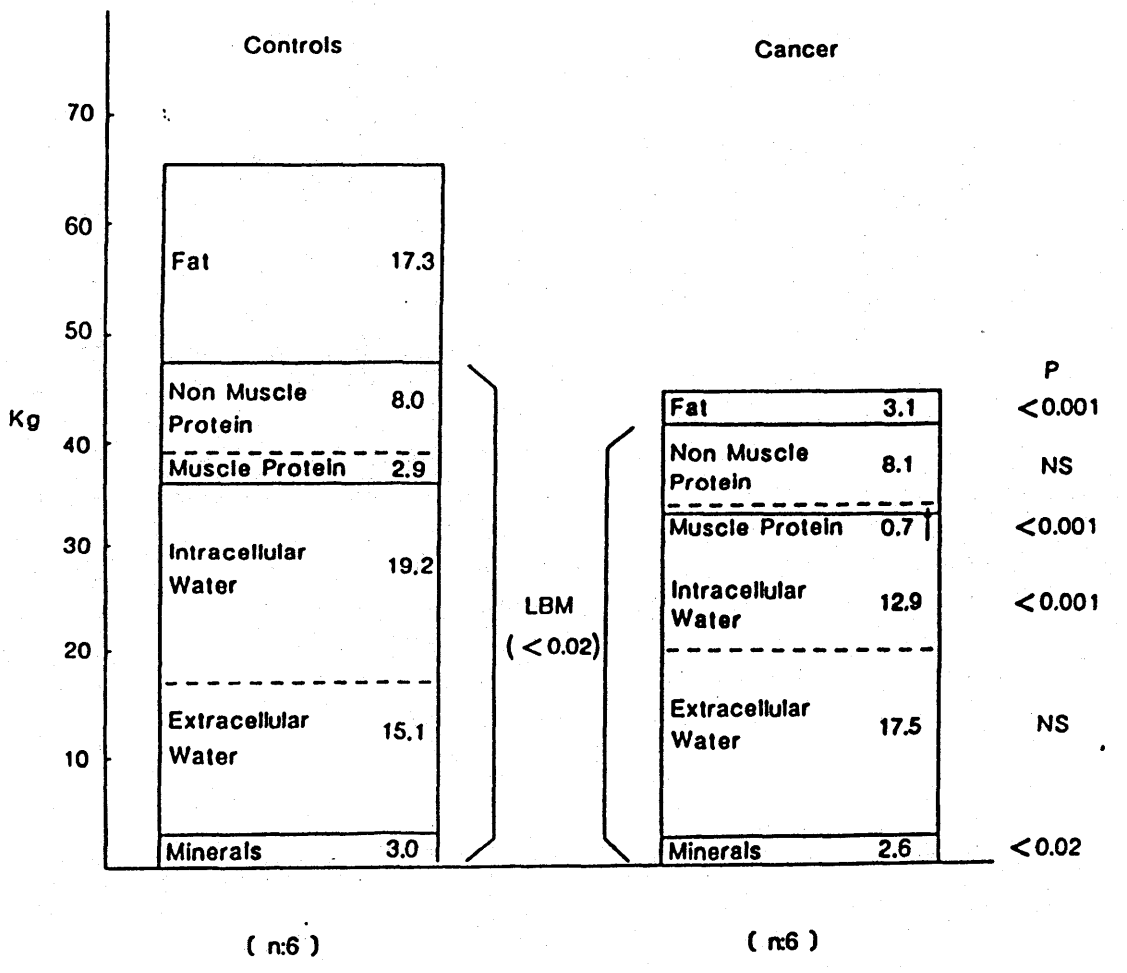


Figure 5.1 Changes in body composition associated with development of cancer cachexia in a group of patients (n = 6) with non-small cell bronchial carcinoma. Neutron activation body composition analysis was carried out on a group of cachectic lung cancer patients and these were compared with a group of healthy individuals who were matched for the age, sex and pre-illness stable weight of the cancer patients.

This study demonstrates clearly that the body composition of cachectic cancer patients is significantly different from that of healthy controls. There was a greater than 80% reduction of body fat and a 13% reduction of lean body mass in a group of cachectic lung cancer patients who had lost 29% of their pre-illness stable weight.

Fat is the major energy reserve in the body. Under normal circumstances when there is an imbalance between energy intake and expenditure the principal body fuel used to correct the deficit is body fat. The fat mass of the cancer patients was 14.2Kg less than that of the weight stable controls (Table 5.6). The energy value of 1Kg of fat is approximately 38MJ. Therefore, to account for the loss of body fat, the total energy deficit of the cancer patients must have been in excess of 532MJ. Since weight loss had occurred over a period of nine months this suggests an average energy deficit of 2MJ/day.

At the time of study the energy intake of the cancer patients was 5MJ less than predicted requirements (Table 5.4). As discussed elsewhere, the measurement of food intake is at best an approximation (Chapter 7; see also Acheson et al., 1980). Nevertheless, a food intake 50% less than predicted requirements would clearly be a major contributing factor to a negative energy balance and to weight loss. However, it cannot be determined whether this reduced food intake was the cause or the result of the cachectic process. The lethargy, depression, lack of mobility and metabolic abnormalities present in cachectic cancer patients may well contribute to anorexia (Calman, 1982). However, the energy deficit of the cancer patients may have been due to an

increased energy expenditure. This mechanism of weight loss is discussed further in Chapter 6.

The protein intake of the cancer patients was reduced by 25% whereas energy intake was reduced by 50% (Table 5.4). This relative preservation of protein intake has been observed in other studies of weight losing cancer patients (Cohn et al., 1981b; Burke, Bryson and Kark, 1980). Whether this contributes to the pattern of altered body composition in cachexia is not known.

The total body nitrogen of the cachectic cancer patients was 19% less than that of the weight stable controls (Table 5.5). In contrast, total body potassium was reduced by more than 33% (Table 5.5). Because muscle has a higher potassium concentration in relation to its protein content than other tissues of the lean body (Burkinshaw, Hill and Morgan, 1978) it is possible to explain this differential loss of potassium by a 76% reduction of muscle protein mass (Table 5.6). An alternative explanation is that there was a general reduction in intracellular potassium and that the overall reduction was not specifically related to loss of potassium rich muscle. However, normal intracellular concentrations of potassium have been demonstrated in the skeletal muscle of both wasted cancer (Symreng, Larrson and Moller, 1985) and non cancer patients (King et al., 1978) and this would tend to suggest that body wasting is not associated with a generalised reduction of body potassium.

Another explanation for the differential loss of potassium is that the patients had lost protein predominantly from the cellular compartment, leaving a relatively high proportion of potassium free

yet nitrogen rich extracellular collagen (James et al., 1984). However, 24h urinary creatinine has been shown to be closely related to body muscle mass (Heymsfield et al., 1983). Therefore, the 69% reduction of urinary creatinine excretion by the cancer patients (Table 5.3) would support the suggestion that there had been a severe loss of muscle mass.

The cause of such muscle wasting in cancer cachexia is unknown. Clearly, the reduced energy and protein intake of the cancer patients (Table 5.4) may have contributed. However, it has been postulated that cancer may specifically reduce the rate of protein synthesis in the skeletal muscle of cachectic patients (Emery et al., 1984a). Whatever the cause, the clinical significance is more obvious. A loss of 75% of body muscle protein would account for the immobility of cachectic patients and would clearly contribute to reduced pulmonary ventilation and the development of fatal pneumonia.

In contrast to simple starvation the non-muscle protein mass of the cachectic patients was not significantly different from that of the healthy controls (Table 5.6). Moreover, the lean body mass was reduced by only 13% (Table 5.6). It has been suggested that the relative preservation of lean tissue (Watson and Sammon, 1980) or more particularly of non-muscle lean tissue (Cohn et al., 1981c) in cancer cachexia may be due to an increased water content of the non-fat mass, the mass of the tumour, the failure of atrophy of visceral organs, or a combination of all three (Heymsfield and McManus, 1985). The results of this study show that the intracellular water space of the cancer patients was reduced whilst the extracellular water space was unchanged (Table 5.6). Furthermore, tumour weight rarely exceeds 1 -

2Kg (Heymsfield and McManus, 1985). Therefore, the most likely explanation for preservation of non-muscle nitrogen would be maintenance of visceral organ mass. This has been documented repeatedly in animal models of cancer cachexia (Yeakel, 1948; Begg and Dickenson, 1951; Morgan and Cameron, 1973) and more recently has been confirmed in humans (Heymsfield and McManus, 1985). The reason for the maintenance of such organs as the liver or spleen are unknown. Increased hepatic protein synthesis has been described in tumour bearing animals and man and it may be that chronic immunological activation with hepatic synthesis of acute phase proteins is a contributory factor (Lundholm et al., 1978; Lundholm et al., 1979). This possibility is discussed further in Chapter 6.

The body mineral content of the cachectic cancer patients was reduced by about 10% (Table 5.6). This was principally due to a 17% reduction in total body calcium (Table 5.5). Since 99% of body calcium is contained in bone, this suggests a reduction of bone mineral mass. The reduced skeletal muscle mass of these patients may have contributed to bone loss since lack of mobility would lead to disuse atrophy.

A group of cachectic lung cancer patients who had lost 29% of their pre-illness stable weight were shown to have lost more than 80% of their body fat and 20% of body protein. The protein loss was accounted for by a 75% reduction of muscle protein mass. Non-muscle protein mass was not affected. This distinguishes cancer cachexia from simple starvation. The mobilization of body fat was sufficient to account for a mean daily negative energy balance of 2MJ/d and loss of body protein indicated a daily negative nitrogen balance of 1.2gN/d. At the time of study daily energy intake was 5MJ/d less than expected and daily nitrogen intake was 2.7gN/d less than expected. These results suggest that a reduced food intake may be a major contributary factor to the altered body composition of lung cancer patients.

CHAPTER 6

The relationship between protein turnover, resting energy expenditure and weight loss in patients with and without cancer

Introduction

Weight loss is most commonly due to a negative energy balance. In cancer patients this could be the result of a decreased food intake, an increased energy expenditure, or a combination of these two. In Chapter 5 it was shown that a reduced food intake was probably a major contributing factor to the continuing protein and energy deficit of cachectic lung cancer patients. This would tend to confirm that when anorexia is present a decreased food intake is probably the major cause of weight loss (Morrison, 1976). However, neither the incidence nor the possible mechanisms of an increased energy expenditure in cancer patients are known (Calman, 1982).

Whole body protein turnover has been estimated to account for between ten and twenty percent of basal energy expenditure in man (Reeds, Fuller and Nicholson, 1985). A reduction in the rate of protein turnover is thought to be one of the main methods of energy conservation during periods of reduced food intake (Waterlow, 1981). It has been suggested that this adaptive mechanism might be impaired in patients with malignant disease (Brennan, 1981) and this possibility is supported by observations that in some cancer patients the rate of whole body protein turnover is elevated above normal (Heber et al., 1982; Jeevanandam et al., 1984; Eden et al., 1984). Thus the energy expenditure of the cancer bearing host might be abnormally high; a situation which could either initiate or worsen a negative energy balance.

Proteins play a crucial role in virtually all biological processes such as enzymatic catalysis, transport and storage, mechanical support and the control of growth and differentiation (Stryer, 1975). In the body, proteins can undergo subtle changes and as the ordered structure of these molecules is critical to their biological function, there is a persistent need for their resynthesis (Reeds, Fuller and Nicholson, 1985).

The continual turnover of protein in the body has several biological advantages. When protein is continually being broken down and resynthesized a small change in either the rate of synthesis or degradation can lead to a substantial change in the amount of that protein. If this protein is a rate limiting enzyme then such protein turnover provides a sensitive mechanism for metabolic regulation. Furthermore, rapid protein turnover enables a limited protein pool to be used with optimal efficiency.

However, protein synthesis is a process which requires energy and some energy may be used in the hydrolysis of peptide bonds when protein is broken down (Waterlow, Garlick and Millward, 1978). Moreover, the reutilization of amino acids is not completely efficient (Waterlow and Stephen, 1966). Thus a state of dynamic protein flux is expensive in terms of energy and to a lesser extent, amino acids.

Not all workers have shown that whole body protein turnover is increased in patients with cancer (Glass, Fern and Garlick, 1983; Emery et al., 1984a). These contradictory results might be explained by the small size and heterogeneity of the groups of patients examined. In addition, few studies have described whole body protein turnover and resting energy expenditure measurments made in the same individuals.

One possible cause of an increased rate of whole body protein turnover in cancer patients might be the production of inflammatory mediators in response to neoplasia. The acute phase protein response is a characteristic alteration in the serum concentration of certain circulatory proteins which usually accompanies the body's inflammatory response to injury (Pepys and Baltz, 1983). Such an acute phase protein response can be demonstrated in the majority of individuals with progressive neoplasia (Cooper and Stone, 1979; Raynes and Cooper, 1983). Furthermore, in patients with trauma or sepsis there is often a marked acute phase response (Pepys and Baltz, 1983) and this is associated with an increase in whole body protein turnover (Kinney and Elwin, 1983).

Thus, the aim of this study was to determine whether rates of whole bodt protein turnover are increased in patients with cancer and, if so whether they are associated with an increased resting energy expenditure and hence weight loss. Whole body protein turnover and resting energy expenditure were measured simultaneously in both weight stable and weight losing patients who had either colorectal cancer or non-small-cell bronchial cancer. These results were compared with those from both weight stable and weight losing non-cancer patients. In addition, rates of whole body protein turnover in cancer patents with an acute phase protein response were compared with those from patients not showing an acute phase response, in order to determine whether an abnormal protein metabolism in patients with cancer is associated with an inflammatory response.

The percentage weight change from pre-illness stable weight was used to divide patients into weight stable (< 5% weight loss) or weight losing groups (> 5% weight loss). This arbitrary division was used to separate those in energy balance from those with a negative energy balance.

Lung cancer patients: Twenty patients with histologically proven, inoperable, non-small cell bronchial carcinoma who were either untreated or who had not undergone antineoplastic therapy within the previous two months underwent standard staging procedures. Investigations included clinical examination, chest X-ray, bone scan, and hepatic ultrasound or isotope scan. Nine were shown to have stage 2 disease and eleven stage 3 disease according to the WHO classification. Eight were weight stable and twelve were weight losing. Of the weight stable group five had stage 2 disease and three had stage 3 disease. In the weight losing group there were four with stage 2 disease and eight with stage 3 disease.

Colon cancer patients: Thirty eight patients with newly diagnosed colorectal carcinoma were all studied prior to laparotomy when histological confirmation and staging according to Dukes' classification was carried out. Sixteen patients had stage B disease, 11 stage C and 10 stage D. Seventeen were weight stable and 21 weight losing. Of the seventeen weight stable patients, 10 were stage B, 4 stage C and 3 stage D. Of the 21 weight losing patients, 7 were stage B, 7 stage C and 7 stage D.

Non-cancer patients: Twenty two patients with various non-neoplastic disease who had been admitted to a surgical ward for routine investigations were used as a control group. Eight were weight stable, and 14 were weight losing. The diagnosis of the 8 weight stable individuals were 4 benign polyp of the colon, 2 duodenal ulcer disease, 1 cholelithiasis and 1 benign stricture of the large intestine. The diagnosis of the 14 weight losing individuals were 6 duodenal ulcer, 2 cholelithiasis, 2 benign strictures of the small intestine, 2 spastic bowel syndrome, 2 benign polyps of colon and 1 caecal diverticulum.

All patients were of performance status 2 or better (World Health Organization Performance Score), had normal adrenal and thyroid functions tests, and were clinically judged to be free of other metabolic or endocrine disorders. None were pyrexial, had clinical or radiological evidence of infection, were receiving steroids or were severely anaemic.

All patients had a normal serum urea and creatinine and although some patients with hepatic metastases had abnormal liver function tests, none had a bilirubin concentration outside the normal range (3 - 18umol/l).

The study was approved by the local ethical committee. All patients were informed of the purpose and procedure of the study, and all gave informed consent.

Measurement of whole body protein turnover: Whole body protein turnover was measured as described in Chapter 2. A primed continuous 24 hour infusion of ^{15}N-glycine was administered and the isotopic enrichment of urinary ammonia and urea was measured in urine collected during the second half of the infusion (12 - 24 hours).

Enrichment of a sample taken at 24 hours was compared with that from the 12 to 24 hour collection to ensure that isotopic equilibrium had been achieved. No significant difference was detected between the ^{15}N enrichement of the integrated 12 - 24 hour sample and that of the sample taken at 24 hours for either urea or ammonia.

Rates of whole body protein turnover, synthesis and breakdown were calculated using the stochastic model of Picou and Taylor Roberts (1969) as described in Chapter 2. Since this study was performed with patients in the fasting state the rate of protein turnover was equal to the rate of protein breakdown.

Measurement of resting energy expenditure: Measurements of resting energy expenditure were made by indirect calorimetry as described in Chapter 3.

Experimental design: Whole body protein turnover and resting energy expenditure were measured simulatenously. On the day prior to study patients ate a standard hospital diet. After an overnight fast, patients remained in bed from the time of wakening. The baseline enrichment of ^{15}N in ammonia and urea was obtained from a urine sample taken at 0800 hours. At 0900 hours, a primed, constant 24 hour infusion of ^{15}N-glycine was commenced. At this time, the patient's head was positioned in the canopy of the indirect calorimeter. Following a 30 minute acclimatisation run there was a 10 minute break. A 40 minute calorimetry run was then undertaken.

During the 24 hour infusion patients were allowed free access to water, but not to food. The priming dose and rate of infusion of ^{15}N-glycine were as described on page 47. During the infusion the

urine was collected in two consecutive 12 hour periods. A further sample was obtained at the end of the 24 hour infusion.

Assessment of acute phase protein status: Serum C-reactive protein was measured in all patients as a marker for the presence of an acute phase protein response (Pepys and Baltz, 1983). Serum taken at the time of the protein turnover study was stored at $-80^{o}C$ until analysis. C-reactive protein was measured by a standard radial immuno-diffusion technique using antisera and standards obtained from Behringwerke AG (Marburg, Lahn, West Germany). The limit of detection of this assay is a C-reactive protein concentration of 5mg/l or less. An undetectable concentration of C-reactive protein was considered to indicate that there was no acute phase protein. A concentration of > 10mg/l was designated as a positive acute phase response (Cooper and Stone, 1979; Raynes and Cooper, 1983).

Statistical analysis: Results were tested for significance by a one way analysis of variance. Individual experimental groups were compared with non-cancer controls using the method described by Dunnett (1955).

Results

Of the weight losing patients, those with lung cancer had lost significantly more weight (21%, $P < 0.05$) than either the colon cancer patients (14%) or the non-cancer patients (14%) (Table 6.1). The mean duration of weight loss was similar for all groups. The serum albumin concentration was within the normal range for all patients except for the weight losing lung cancer patients where the concentration (32 ± 2g/l) was significantly less than that of the weight stable lung cancer patients (37 ± 1g/l $P < 0.05$).

	NON-CANCER		LUNG CANCER		COLON CANCER	
	WS*	WL**	WS	WL	WS	WL
Number	8	14	8	12	17	21
Age (yr)	59 ± 4	58 ± 5	62 ± 4	58 ± 4	65 ± 2	64 ± 3
Weight (Kg)	72 ± 6	57 ± 3	67 ± 4	53 ± 5	60 ± 3	58 ± 2
% weight loss	1 ± 1	14 ± 2	0	21 ± 3	1 ± 1	14 ± 2
Duration of weight loss (m)	1 ± 1	6 ± 1	0	7 ± 1	1 ± 1	6 ± 1
Albumin	40 ± 1	38 ± 1	37 ± 2	32 ± 2	38 ± 2	37 ± 1

* WS : Weight Stable
** WL : Weight Losing

Table 6.1 Clinical details of patients undergoing measurement of whole body protein turnover and resting energy expenditure.

Values are mean ± SEM.

Whole body protein synthesis rates: The rates of whole body protein synthesis and degradation of weight stable and weight losing lung cancer, colon cancer and non-cancer patients are shown in Table 6.2. When the isotopic enrichment of urinary ammonia was used to calculate rates of protein synthesis the mean rate in all groups of cancer patients was at least 75% greater than that of weight stable non-cancer controls ($P < 0.05$). However, although the mean rate of protein synthesis in weight losing non-cancer patients was greater than that of weight stable controls this difference was not statistically significant ($P > 0.05$). Weight stable and weight losing lung cancer patients had slightly, but not significantly, higher mean rates of protein synthesis compared with weight stable and weight losing colon cancer patients (31% and 16% respectively). Protein synthesis rates based on the isotopic enrichment of urinary urea were between 25 and 52% greater than those calculated from the isotopic enrichment of urinary ammonia. However, the overall pattern of results was similar to that obtained with ammonia.

Whole body protein degradation rates: The rates of protein degradation (equivalent to protein turnover : for explanation see Materials and Methods) were all greater than the corresponding rates of protein synthesis since measurements were made with patients in the post absorptive state (Table 6.2). The mean rates of protein degradation, based on the isotopic enrichment of urinary ammonia, for all groups of cancer patients were more than 60% greater than those of the weight stable non-cancer controls ($P < 0.05$). However, although the mean rate of protein degradation in weight losing non-cancer patients was greater than that of weight stable controls this difference was not statistically significant ($P > 0.05$).

		NON-CANCER		LUNG CANCER		COLON CANCER	
		WS*	WL**	WS	WL	WS	WL
PROTEIN SYNTHESIS	ammonia	1.75 ± 0.19	2.98 ± 0.42	4.11 ± 0.65	4.04 ± 0.65	3.13 ± 0.39	3.49 ± 0.30
(gP/Kg/d)	urea	2.66 ± 0.34	4.10 ± 0.51	5.14 ± 0.51	5.44 ± 0.72	4.10 ± 0.39	4.40 ± 0.37
PROTEIN DEGRADATION	ammonia	2.19 ± 0.19	3.48 + 0.43	4.61 ± 0.70	4.71 ± 0.56	3.63 ± 0.40	4.00 ± 0.29
(gP/Kg/d)	urea	3.09 ± 0.34	4.61 ± 0.52	5.65 ± 0.57	6.13 ± 0.75	4.58 ± 0.41	4.92 ± 0.37

* WS : Weight Stable
** WL : Weight Losing

Table 6.2 Whole body protein kinetics of cancer and non-cancer patients. Protein synthesis and degradation rates are expressed with reference to the enrichment of either ammonia or urea. Protein degradation rates are equivalent to protein turnover rates (For explanation see Materials and Methods). Values are mean ± SEM.

Protein degradation was slightly, but not significantly, higher in weight stable and weight losing lung cancer patients compared with weight stable and weight losing colon patients (27% and 18% respectively). The mean rates of protein degradation were 16% - 30% higher when calculated from the isotopic enrichment of urinary urea, but the pattern of results was similar to that obtained with urinary ammonia.

Resting energy expenditure: Table 6.3 shows the rates of resting energy expenditure and whole body protein turnover expressed with reference to the patients' lean body mass. There were no significant differences between the rates of resting energy expenditure of the weight stable non-cancer controls and any of the groups examined. The mean rate of whole body protein turnover was significantly greater in lung cancer patients ($P < 0.05$) but not in colon cancer patients or weight losing non-cancer patients when compared with the weight stable controls. There was no correlation between individual rates of whole body protein turnover and rates of resting energy expenditure (Figure 6.1). Moreover, there was no correlation between individual rates of protein turnover and the severity of patients' weight loss (Figure 6.2).

Acute phase protein response

An acute phase protein response was detected in the majority of cancer patients (41/58). However, there were no statistically significant differences in the rates of whole body protein synthesis or turnover between those with or without an acute phase protein reaction (Table 6.4).

		NON-CANCER		LUNG CANCER		COLON CANCER	
		WS*	WL**	WS	WL	WS	WL
WHOLE BODY PROTEIN TURNOVER							
(gP/KgLBM/d)	ammonia	3.05 ± 0.29	4.27 ± 0.55	5.80 ± 0.90	5.46 ± 0.64	5.21 ± 0.68	4.78 ± 0.38
	urea	4.29 ± 0.49	5.70 ± 0.64	7.10 ± 0.69	7.10 ± 0.84	6.50 ± 0.70	5.89 ± 0.48
RESTING ENERGY EXPENDITURE (KJ/KgLBM/d)		119 ± 5	118 ± 2	122 ± 5	129 ± 5	132 ± 8	119 ± 4

* WS : Weight Stable

**WL : Weight Losing

Table 6.3 Whole body protein turnover and resting energy enxpenditure of cancer and non-cancer patients. Both protein turnover and resting energy expenditure are expressed with reference to the patients lean body mass. Results are mean ± SEM.

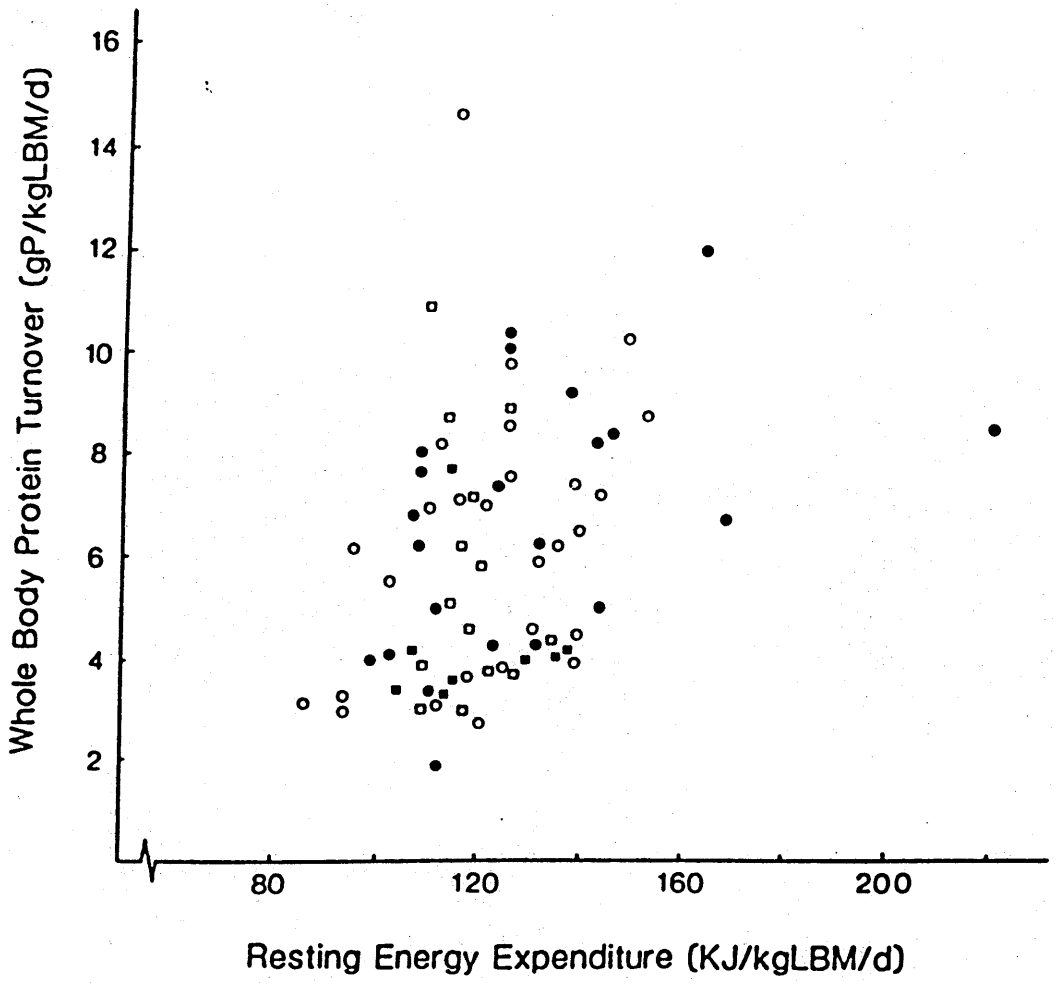


Figure 6.1 Whole body protein turnover plotted against resting energy expenditure in cancer patients (weight stable : ● , weight losing: O), and non-cancer patients (weight stable: ■ , weight losing: □). Whole body protein turnover was calculated from the isotopic enrichment of urinary urea and both variables are expressed with reference to the patients lean body mass.

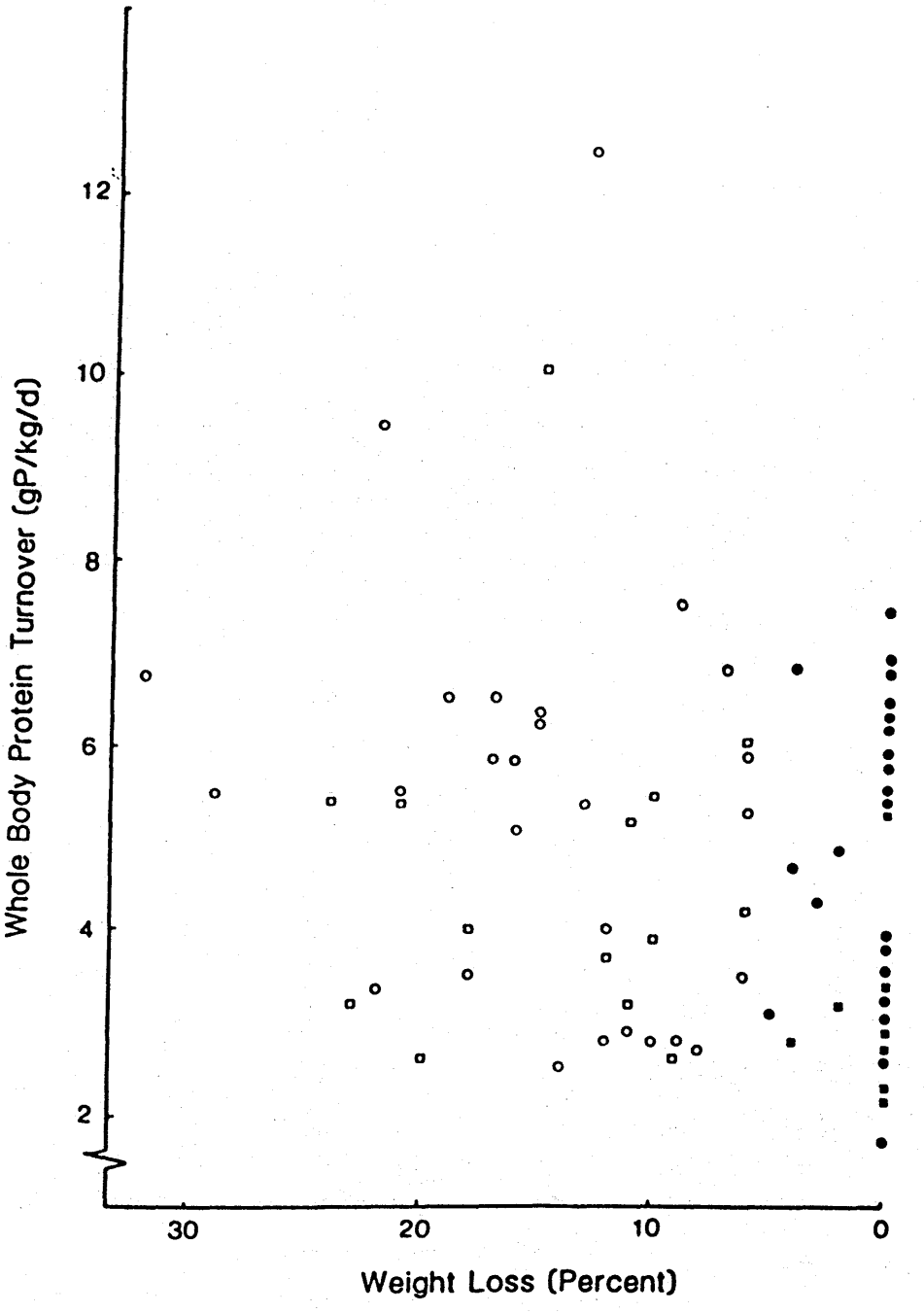


Figure 6.2 Whole body protein turnover of cancer and non-cancer patients plotted against percentage weight loss (For key see Figure 6.1).

Acute Phase Protein Response	-ve(CRP<5mg/l)	+ve(CRP>10mg/l)
n	17	41
Whole Body Protein Synthesis (gP/Kg/d)	4.02 ± 0.39	4.88 ± 0.30
Whole Body Protein Degradation (gP/Kg/d)	4.49 ± 0.41	5.41 ± 0.31

Table 6.4 Whole body protein kinetics of cancer patients with or without an acute phase protein response. An acute phase protein response was defined as a concentration of C-reactive protein > 10mg/l in the patients serum. Values are mean ± SEM.

The results of this study demonstrate clearly that patients with lung or colon cancer, whether weight stable or weight losing, tend to have an elevated rate of whole body protein turnover. When whole body protein turnover was expressed with reference to total body weight patients with either lung or colorectal cancer had turnover rates which were 50 - 70% higher compared with weight stable non-cancer controls ($P < 0.05$). These elevated rates of whole body protein turnover were not related to altered body composition since the rate of turnover was increased in both weight stable and weight losing cancer patients. When whole body protein turnover was expressed with reference to lean body mass the mean rate in patients with lung cancer was significantly greater than that of weight stable non-cancer controls ($P < 0.05$). However, although the mean rate in colon cancer patients was approximately 50% higher than weight stable non-cancer controls, this difference was not significant ($0.1 < P > 0.05$). This suggests that there was a greater elevation of protein turnover in lung cancer patients when compared with colon cancer patients. A higher rate of protein turnover in the lung cancer group might have been due to a greater proportion of lung cancer patients having advanced disease.

It has been suggested that elevated whole body protein turnover may increase the cancer patient's energy expenditure and thus initiate or worsen a negative energy balance (Eden et al., 1984). In this study there was no difference between the rates of resting energy expenditure of cancer patients and that of the weight stable non-cancer controls (Table 6.3). Furthermore, there was no correlation between the rates

of whole body protein turnover and resting energy expenditure for any of the patient groups examined (Figure 6.1). Thus, although patients with malignant disease had an elevated rate of whole body protein turnover, this was not associated with a detectable increase in their resting energy expenditure.

An elevated rate of whole body protein turnover was observed in all cancer patients, yet only a proportion lost weight. Therefore, the suggestion that cancer patients lose weight because of an increase rate of resting energy expenditure or whole body protein turnover (Jeevanadam et al., 1984) must be an oversimplification.

The weight stable cancer patients had significantly higher rates of whole body protein turnover than the weight stable non-cancer controls (Table 6.2). This increase occurred in the absence of trauma or sepsis, the two main pathological states normally associated with increased whole body protein flux (Kinney and Elwyn, 1983). The rate of protein synthesis in human tumours is approximately the same as that in the tissue of origin (Stein et al., 1982b), and human neoplasms rarely exceed 1% of body mass (Stein, 1982a). Thus, it is unlikely that protein synthesis within the tumour itself could have caused the observed increase in whole body protein turnover. It was decided, therefore, to investigate whether an inflammatory response by the host might be contributing to accelerated protein flux.

The majority of cancer patients in this study had a positive acute phase protein reaction (Table 6.4). Although the turnover rates in cancer patients with an acute phase protein response were 20%

greater than in those with no response this difference was not significant. However, the possibility that the immune response of the host may participate in the production of some of the metabolic abnormalities of the cancer host is worthy of further investigation.

The rate of protein turnover has been shown to decrease during uncomplicated starvation (Jeevanandam, 1984; Rose et al., 1983) and it has been proposed that this may be a mechanism of energy conservation. Since anorexia and the associated decreased food intake is a major cause of weight loss in cancer patients (Morrison, 1976; see also Chapter 5) it might be expected that protein turnover should decrease with increasing weight loss. The results of this study indicate that in patients with either lung or colorectal cancer there is no correlation between whole body protein turnover and weight loss (Figure 6.2). This emphasises the contrast between the metabolism of simple starvation and that of cancer cachexia.

It is possible that a small rise in resting energy expenditure did occur in the cancer patients, but that this was not detected by the methods that were used. From stoichiometry it can be estimated that the minimum energy required for the synthesis of a gram of protein is about 3.6KJ (Reeds, Fuller and Nicholson, 1985). Using the isotopic enrichment of urinary urea to calculate rates of protein synthesis, the mean rate of protein synthesis for all cancer patients was 4.6gP/Kg/d compared with 2.7gP/Kg/d for controls. This represents an increase of 1.9gP/Kg/d. Since the weight of the patients was about 60Kg, the cancer patients were synthesising an extra 114g of protein per day. This extra protein synthesis would require the expenditure of 410KJ which represents less than 8% of the patients' mean resting energy expenditure. A change of this magnitude would be at the limit of

detection of the method we used to measure resting energy expenditure (Chapter 3, page 54). Moreover, resting energy expenditure can vary between normal individuals by up to 50% (Garrow, 1985). Thus, if the above minumum estimates for the energy cost of protein synthesis are correct, we would probably not have detected the expected increase in resting energy expenditure. Nevertheless, an increase of energy expenditure of this amount lasting a period of months would be highly. significant in terms of a patient's cumulative energy deficit.

That the methods which were used were unable to detect a small rise in resting energy expenditure is one way to explain why the resting energy expenditure of the cancer patients was apparently not affected by an increased rate of whole body protein turnover. An alternative explanation could be that since resting energy expenditure is the sum of all energy dependent processes in the resting, post absorptive individual, other energy requiring processes were reduced to compensate for the energy cost of increased protein turnover. It is also possible that although the patients with cancer had an elevated rate of tracer flux, this might represent regional changes in protein turnover rather than a uniformly elevated rate of protein turnover in the whole body. It has been demonstrated that protein synthesis can be depressed in skeletal muscle and elevated in the liver of both patients and animals with cancer cachexia (Emery et al., 1984; Lundholm et al., 1978). Furthermore, the efficiency of energy conservation and of protein synthesis may vary from one tissue to another. Thus, because whole body protein turnover and resting energy expenditure are measurements derived from all tissues of the body it would be wrong to assume that these two variables should necessarily change in parallel.

The hypothesis that cancer patients have an elevated rate of whole body protein turnover and that this leads to an increase in resting energy expenditure and to weight loss was examined. Rates of whole body protein turnover and resting energy expenditure were measured simultaneously in weight stable and weight losing patients with lung (n = 20) or colorectal cancer (n = 38) and these were compared with weight stable and weight losing non-cancer controls (n = 22).

The cancer patients synthesised on average 1.9gP/Kg/d more protein than the weight stable controls. However, the resting energy expenditure of cancer patients was not significantly greater than that of the non-cancer controls. Moreover, there was no correlation between individual rates of whole body protein turnover and resting energy expenditure or weight loss.

Thus, although cancer patients had rates of whole body protein turnover which were 50 - 70% greater than controls, this was not associated with an increased energy expenditure, and it did not necessarily lead to weight loss. The assumption that elevation of whole body protein turnover or resting energy expenditure causes weight loss in cancer patients must be an over simplification. The nature of the negative energy balance in these patients remains to be determined.

CHAPTER 7

Mechanisms of weight loss in two contrasting rodent models of cancer cachexia

Introduction

The mechanisms of weight loss in cancer cachexia are not clearly understood. For some patients the primary phenomenon is a lack of food intake (Morrison, 1976; see also Chapter 5). In contrast, other patients may have an adequate energy and protein supply with no apparent intestinal malfunction and yet continue to lose weight (Heber, Byerly and Chlebowski, 1985). Certain abnormalities of protein, fat and carbohydrate metabolism and an increased energy expenditure have been proposed to account for such weight loss (Brennan , 1977; Lawson et al., 1982). However, it is not known whether the metabolic abnormalities described in cachectic cancer patients are the cause or the result of their cachexia. Furthermore, accurate documentation of food intake in humans is extremely difficult (Acheson et al., 1980). Thus, it is often impossible to determine what contribution any metabolic abnormality has made to a patient's negative energy or protein balance. Numerous different factors may contribute to weight loss in each cancer patient and each of these factors may act to a variable degree from one patient to another. Thus, obtaining a uniform group of patients in which to evaluate a particular mechanism of weight loss is extremely difficult. Moreover, investigation of human cachexia is severely limited by ethical considerations.

These problems have prompted the search for an appropriate animal model of human cachexia. Unfortunately most models involve rodent tumours with doubling intervals between 12 - 48 hours, where signs of

cachexia develop only when the tumour reaches 30 - 40% of total body weight (Begg and Dickinson, 1951; Recheigl, Grantham, and Greenfield, 1961). In contrast, human tumours grow much more slowly and tumour burden rarely exceeds 5% of body weight (Heymsfield and McManus, 1985). It is therefore not surprising that animal models have, as yet, proven to be of limited value in the investigation of human cachexia (Lundholm et al., 1978).

The Walker 256 carcinosarcoma has a doubling interval of about 36 hours when grown in Sprague - Dawley rats and can reach 20% of host body weight before causing significant weight loss (Begg and Dickinson, 1951). The later stage of tumour growth is also associated with marked anorexia (Guaitani et al., 1982). Alternatively, the MAC-16 is a recently described, slow growing, transplantable adenocarcinoma of the colon which produces weight loss in syngeneic mice when tumour size is less than 3% of host weight (Tisdale, Brennan and Fearon, 1985). On this basis the MAC-16 tumour might be a more representative model of human cachexia. Moreover, the mechanisms of weight loss in this model might give valuable insight into that of humans with cancer.

The present study was undertaken to determine the mechanisms of weight loss in rats bearing the Walker 256 carcinosarcoma. These results are compared with those obtained from a similar study carried out on mice bearing the MAC-16 adenocarcinoma. Body composition analysis was performed in both models in order to determine the nature of host tissue loss and the contribution of the tumour mass itself to host wasting. This study is described in Part 1.

When tumour mass represented 10% of the host, the body composition and weight of rats bearing the Walker 256 tumour was

unaltered except that there had been a transfer of protein and water from the host to the tumour. However, in mice bearing the MAC-16 tumour neither the mass or composition of the tumour could account for host weight loss. This pattern resembles that observed in human cachexia (see Chapter 5). Therefore food intake and energy expenditure were measured in mice bearing the MAC-16 tumour to determine whether a decreased food intake, an increased energy expenditure or a combination of the two was responsible for weight loss. As discussed in Chapter 6, an elevated protein synthesis rate has been proposed to account for an inappropriate energy expenditure in the cancer host. In order to investigate this possibility rates of protein synthesis were determined in the liver, kidney, heart and skeletal muscle of normal mice and mice bearing the MAC-16 tumour. The results of these studies are presented and discussed in Part 2.

Part 1. Changes in body composition of rats bearing the Walker 256 tumour and of mice bearing the MAC-16 tumour.

Materials and Methods

Animals and Diet: Female Wistar rats and male NMRI mice aged between 8 - 10 weeks were maintained in conditions of controlled temperature and lighting ($21^{o}C \pm 1^{o}C$, 12/12h light/dark cycle). Except where stated in the text, animals were fed ad libitum on a standard diet (CRM diet, Labsure, Rank-Hovis-McDougal, Agricultural Division, Dorset, England). It contained 18.5% protein, 2.5% fat, 56% carbohydrate, 4.3% fibre, 2.4% minerals, 15.0% moisture and added vitamins and trace elements. The gross energy content of the diet was 14.8KJ/g.

Tumours: The rapidly growing Walker 256 carcinosarcoma was obtained from the Institute of Cancer Research, Sutton, Surrey and maintained by routine passage every 14 days in Wistar rats. The tumour arose spontaneously from the mammary pad of a pregnant female rat and has been passaged since 1928 in various non-syngeneic hosts (Fisher and Fisher, 1960). The MAC-16 adenocarcinoma was obtained from Dr J. Double, Clinical Oncology Unit, University of Bradford. The tumour had been chemically induced in the colon of NMRI mice following prolonged administration of 1,2 dimethylhydrazine (Double and Ball, 1975). Viable tumour fragments (100mg) were transplanted subcutaneously into the right flank under aseptic conditions and light ether anaesthesia. The doubling time of the Walker tumour was 36 hours, whilst that of the MAC-16 tumour was 6 days.

Histology: Tissues for light microscopy were fixed in buffered 10% formalin and stained with haematoxylin and eosin. One mm cubes of tumour for electron microscopy were immediately fixed in a 2% solution of glutaraldehyde in Sarasens phosphate buffer. The samples were then treated with 2% osmium tetroxide and dehydrated with ethyl alcohol and propylene oxide. Tissues were embedded in araldite resin prior to sectioning for electron microscopy.

Body composition analysis: Total body nitrogen and phosphorous was analysed by neutron activation analysis (See Chapter 4). Induced radioactivity was measured in a modified clinical whole body monitor after bilateral iradiation with 14 MeV neutrons. The radiation dose equivalent was 116 mSv. This non-destructive procedure allows sequential measurements *in vivo* and compares well with chemical analysis (Preston et al., 1985). Total body water was measured by freeze-drying animal carcasses until constant weight. Water content was calculated from the difference between wet and dry weights.

Lean body mass was assumed to consist of the sum of total body protein (N(g) x 6.25), water and minerals (P(g) x 6) (Preston et al., 1985). Fat mass was obtained by subtraction of lean body mass (g) from total body weight (g).

Experimental Design

Walker 256 carcinosarcoma in rats: Body composition analysis was performed in vivo on a group of rats (n = 6) immediately prior to implantation of the Walker 256 tumour. Fourteen days later animals were sacrificed, tumours were excised and weighed. Neutron activation analysis of both carcasses and tumours was then repeated. The water content of the carcasses and tumours was also measured.

MAC-16 adenocarcinoma in mice: Two groups of NMRI mice were matched for body weight. One group (n = 6) underwent tumour implantation and the other (n = 6) sham operation. Twenty four days later animals were sacrificed, tumours excised, weighed and body composition determined.

Statistical analysis: Where appropriate, Student's T-test for paired or unpaired data was used to determine statistical significance.

Results

Histology

Walker 256 tumour: Light microscopy revealed the Walker 256 to be a very poorly differentiated tumour (plate 7.1). Large areas of haemorrhagic necrosis were associated with an inflammatory infiltrate. Cells were round or polygonal and were arranged in sheets. The nucleus

to cytoplasm ratio was high and frequent mitotic figures, often with an abnormal configuration, were present.

Electron microscopy confirmed the loose arrangement of cells in sheets (Plate 7.2). Nuclei were large and frequently exhibited an irregular contour with invaginations. Nucleoli were large and often multiple. The cytoplasm contained relatively few organelles (Plate 7.3). Mitochondria were round or oval and had poorly organised cristae or were empty. The Golgi apparatus of the cells was not a prominant feature. Smooth and rough endoplasmic reticulum was scanty. Numerous cytoplasmic vacuoles were present. Apposition of cells appeared for the most part as a simple contact of cytoplasmic borders. No junctional complexes were observed.

MAC-16: Light microscopy revealed the MAC-16 to be a moderately well differentiated adenocarcinoma which developed a necrotic core as it enlarged (Plate 7.4). The tumour was associated with an intense inflammatory infiltrate which extended from the surrounding subcutaneous tissues into the necrotic core. Tumour cells were mostly arranged in acini (Plate 7.5), though in some areas little acinar differentiation was evident. Within an acinus, tumour cells had a columnar appearance. Large, round, vesicular nuclei with prominant nucleoli were situated near the abluminal membrane. Numerous mitotic figures often with an abnormal configuration were present (Plate 7.5).

Electron microscopy again demonstrated the acinar arrangement of tumour cells (Plate 7.6). Nuclei were large and the nuclear chromatin was finely dispersed. Well developed nucleoli containing electron dense granules as well as vacuoles were present. The cytoplasm was densely packed with organelles (Plate 7.7). Mitochondria were round

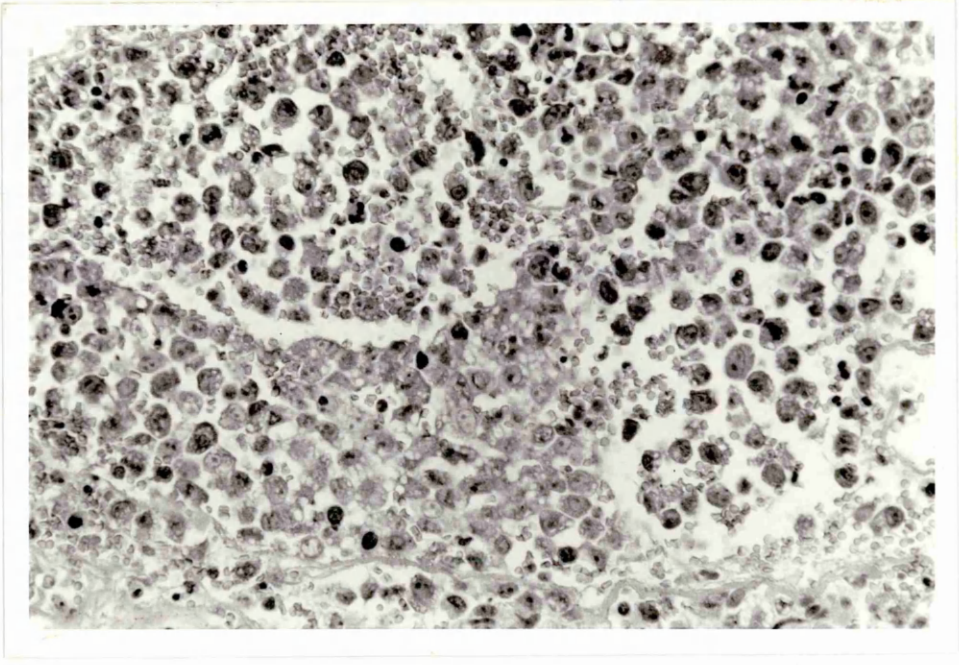


Plate 7.1 Photomicrograph of Walker 256 tumour showing pleomorphic tumour cells arranged in sheets. Poor cell to cell adhesion and extensive haemorrhage are features. (Magnification x 40).

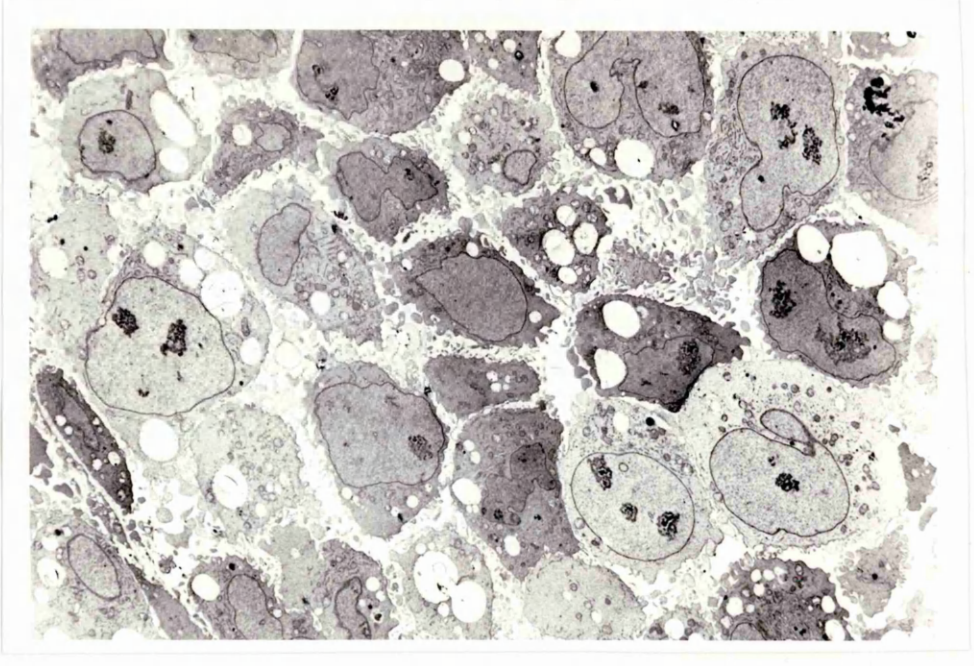


Plate 7.2 Electronmicrograph of Walker 256 tumour showing loose arrangement of tumour cells, high nucleus to cytoplasm ratio and irregularity of nuclear membrane. (Magnification x 1820).

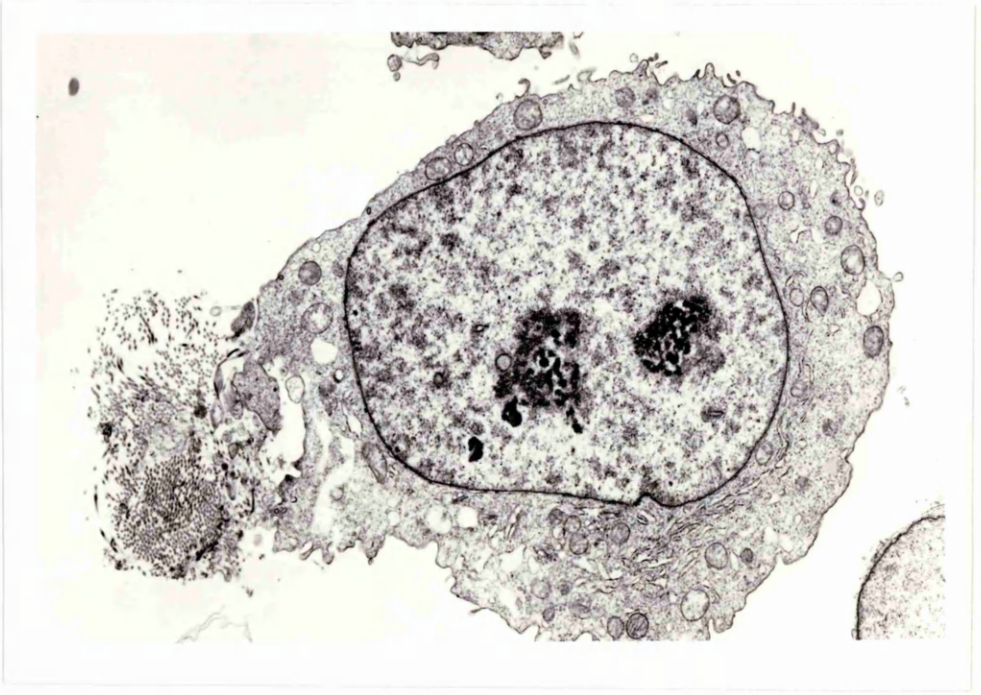


Plate 7.3 Electronmicrograph of Walker 256 tumour cell showing lack of intracellular organelles, numerous cytoplasmic vacuoles and poorly formed mitochondrial cristae. (Magnification x 6020).

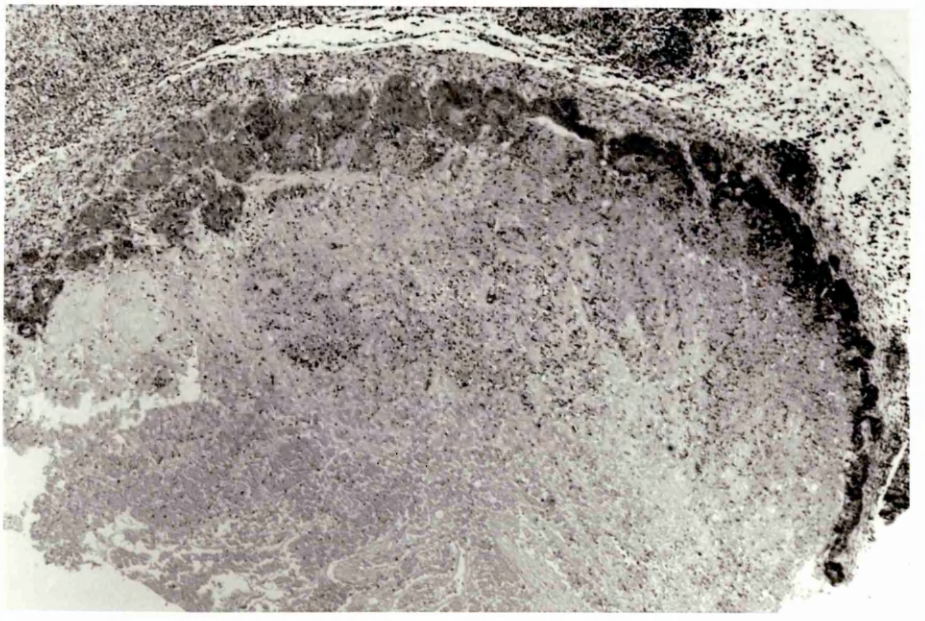


Plate 7.4 Photomicrograph of MAC-16 tumour showing rim of viable tumour cells surrounding a necrotic core. An inflammatory infiltrate extends from the surrounding subcutaneous tissues into the necrotic core of the tumour. (Magnification x 4)

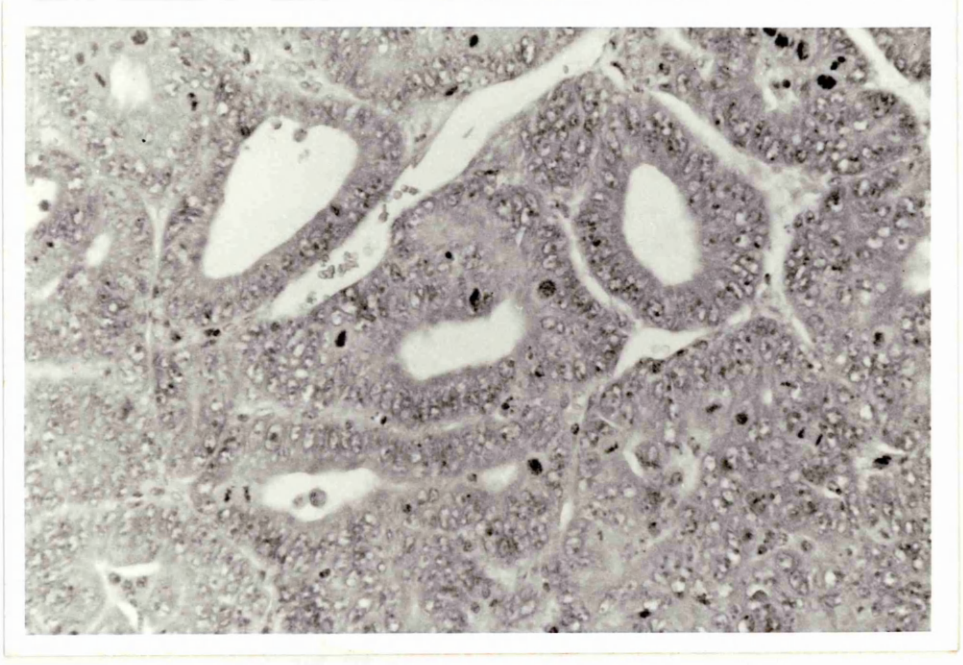


Plate 7.5 Photomicrograph of MAC-16 tumour showing acinar arrangement of columnar cells. Numerous mitotic figures are present. (Magnification x 40).

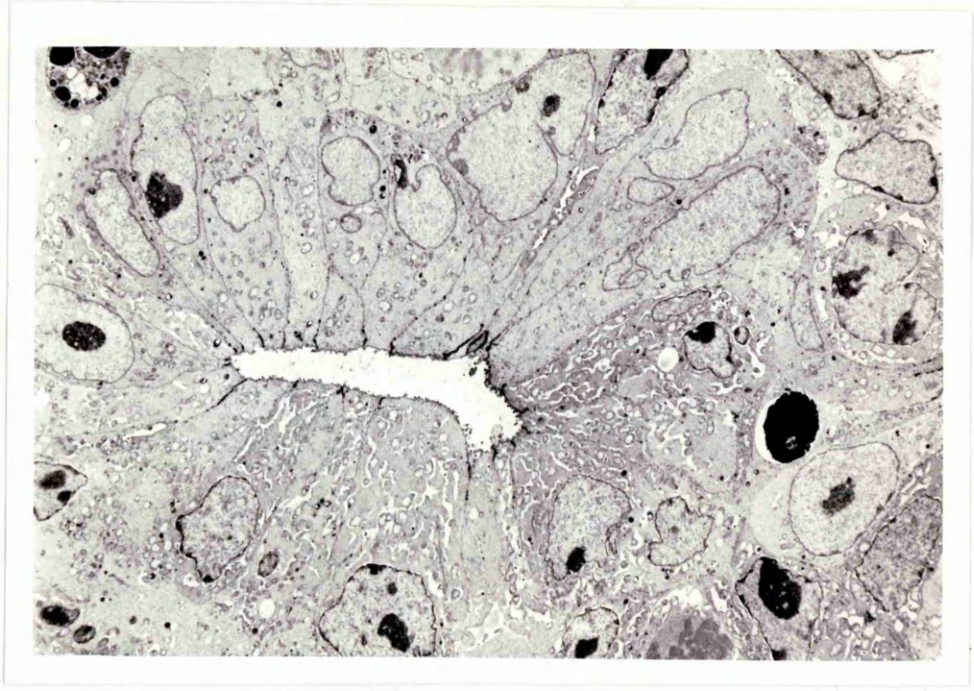


Plate 7.6 Electronmicrograph of MAC-16 tumour showing acinar arrangement of tumour cells. The columnar shaped cells are closely apposed to each other and numerous junctional complexes are present near the lumen of the acinus. (Magnification x 5040).

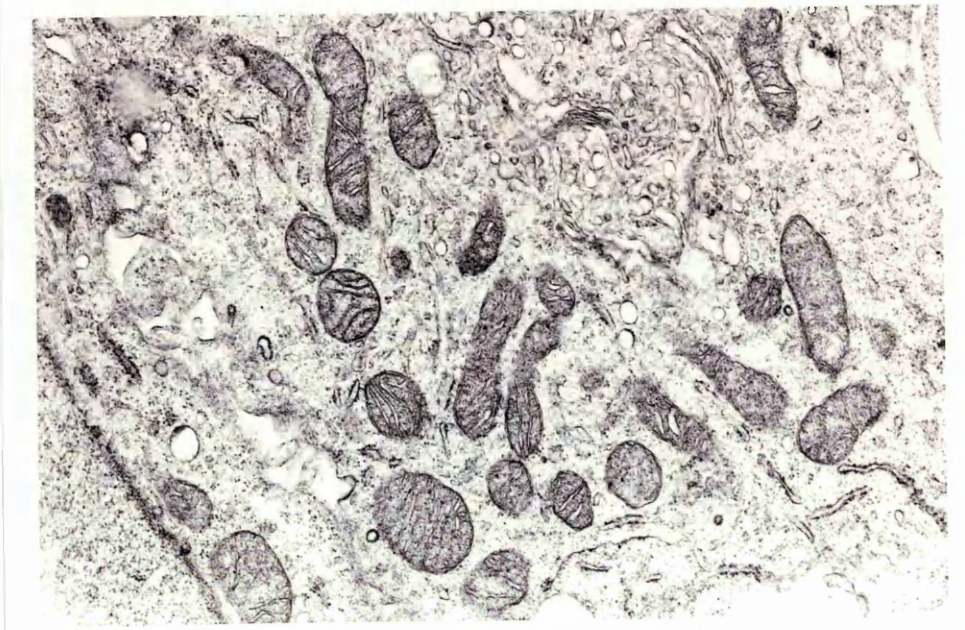


Plate 7.7 Electronmicrograph of MAC-16 tumour showing detail of mitochondria and Golgi apparatus. Mitochondria have well formed cristae. (Magnification x 17640).

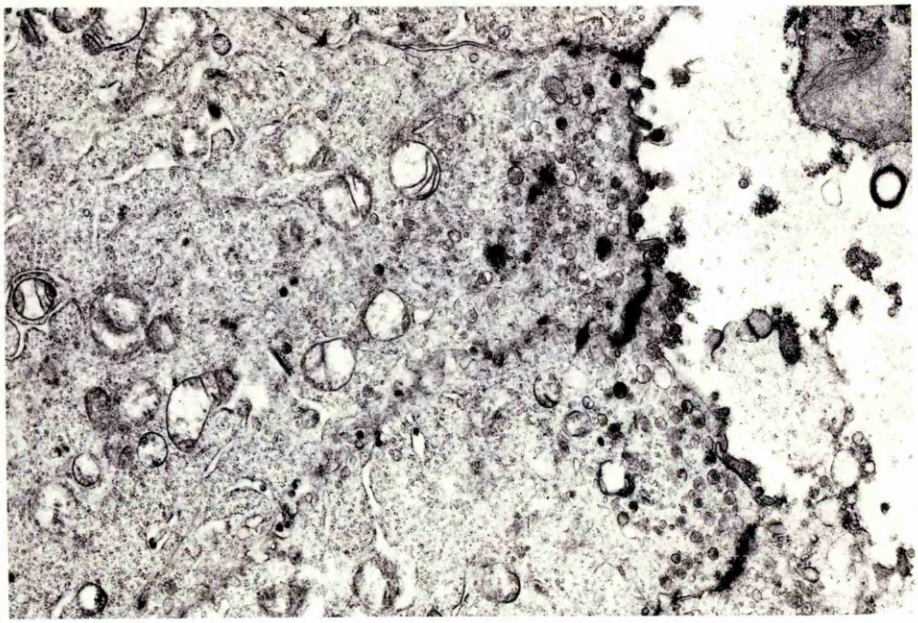


Plate 7.8 Electronmicrograph of MAC-16 tumour showing the luminal surface of the tumour cells. The cells are joined by junctional complexes and on the luminal surface microvilli are present. Numerous membrane bound vesicles are present just below the luminal surface. (Magnification x 28600).

to ovoid and generally had well formed cristae. However, in some sections the mitochondria were empty. Most cells had a well developed Golgi apparatus (Plate 7.7) and some rough endoplasmic reticulum. Numerous free ribosomes were scattered throughout the cytoplasm.

Each tumour cell was closely opposed to its neighbour and towards the lumen of the acinus cells were joined by junctional complexes (i.e. tight junctions and desmosomes ; Plate 7.8). Irregular microvilli were present on the luminal surface and, in some cells, numerous membrane bound vesicles were present in the subjacent cytoplasm (Plate 7.8).

Changes in body composition of rats bearing the Walker 256 tumour: The body composition of rats both before and 14 days after implantation of the Walker 256 tumour is shown in Figure 7.1. The total body weight (190.7 $\pm$ 3.1 cf 190.5 $\pm$ 6.1g : mean $\pm$ s.e.m.), fat mass (21.8 $\pm$ 0.9 cf 20.0 $\pm$ 2.1g), mineral mass (8.3 $\pm$ 0.2 cf 7.6 $\pm$ 0.2g), protein mass (37.0 $\pm$ 0.6 cf 36.5 $\pm$ 0.9g) and total body water (123.0 $\pm$ 2.1 cf 126.4 $\pm$ 3.9) before and after tumour growth were not significantly different. The tumour mass itself contained 3.3 $\pm$ 0.2g protein, 18.5 $\pm$ 1.0g water and represented 10% of total body weight.

Changes in body composition of mice bearing the MAC-16 tumour: The body composition of mice bearing the MAC-16 tumour and of non-tumour bearing controls are shown in Figure 7.2. The mean weights of the two groups at the start of the study were not significantly different (35.5 $\pm$ 2.0 cf 36.0 $\pm$ 1.2g). Twenty four days after tumour implantation, the tumour bearing mice had lost 13% of their total body weight and this was significantly reduced compared with controls (31.4 $\pm$ 1.1 cf 36.3 $\pm$ 2.0g : $P < 0.02$). Total body water (22.4 $\pm$ 0.05 cf 24.1 $\pm$ 0.5g) and mineral mass (1.0 $\pm$ 0.1 cf 1.1 $\pm$ 0.1g) were not significantly decreased.

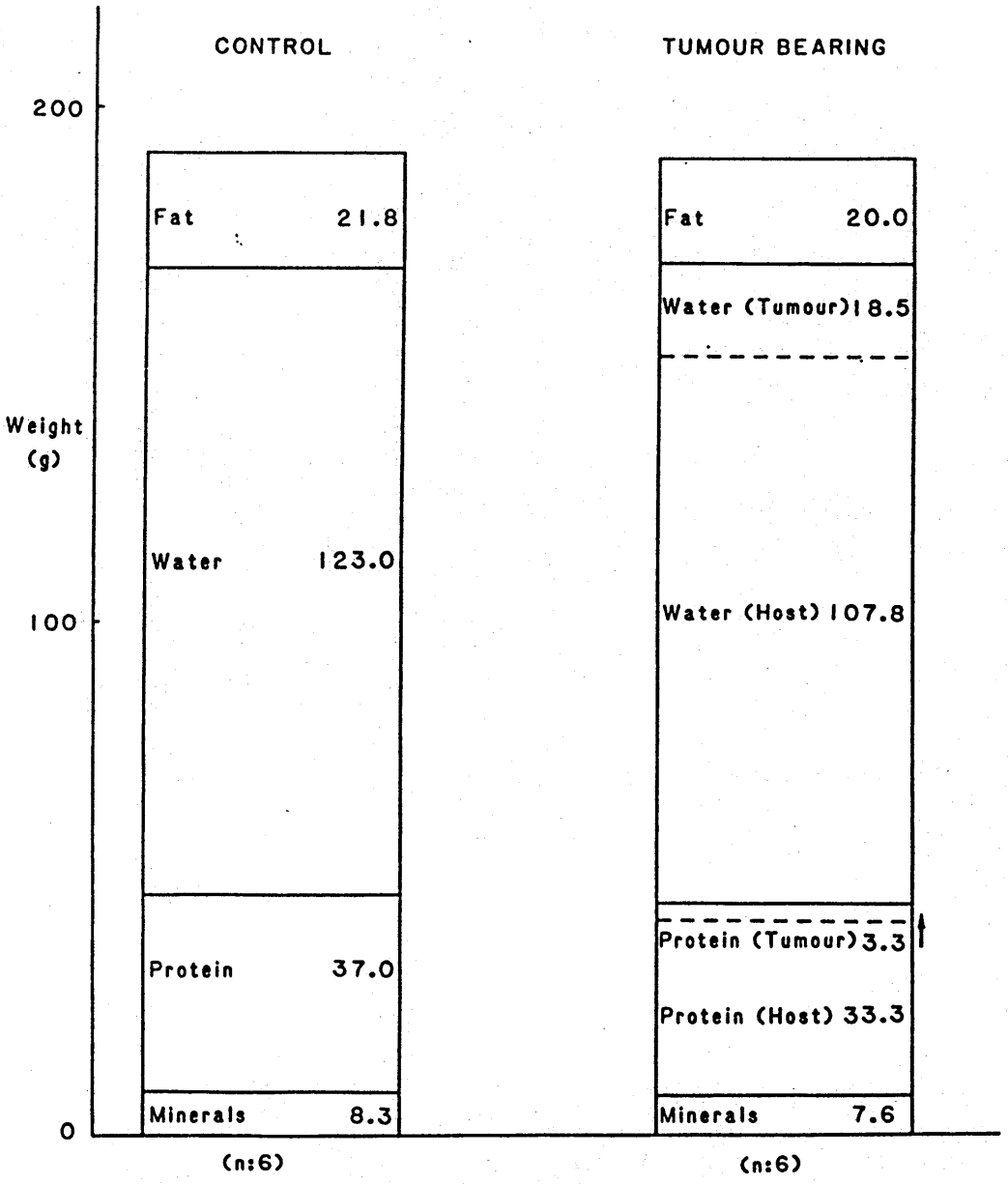


Figure 7.1 Changes in body composition of rats bearing the Walker 256 tumour. Neutron activation body composition analysis in vivo was performed before tumour implantation and repeated after 14 days when the animals were sacrificed.

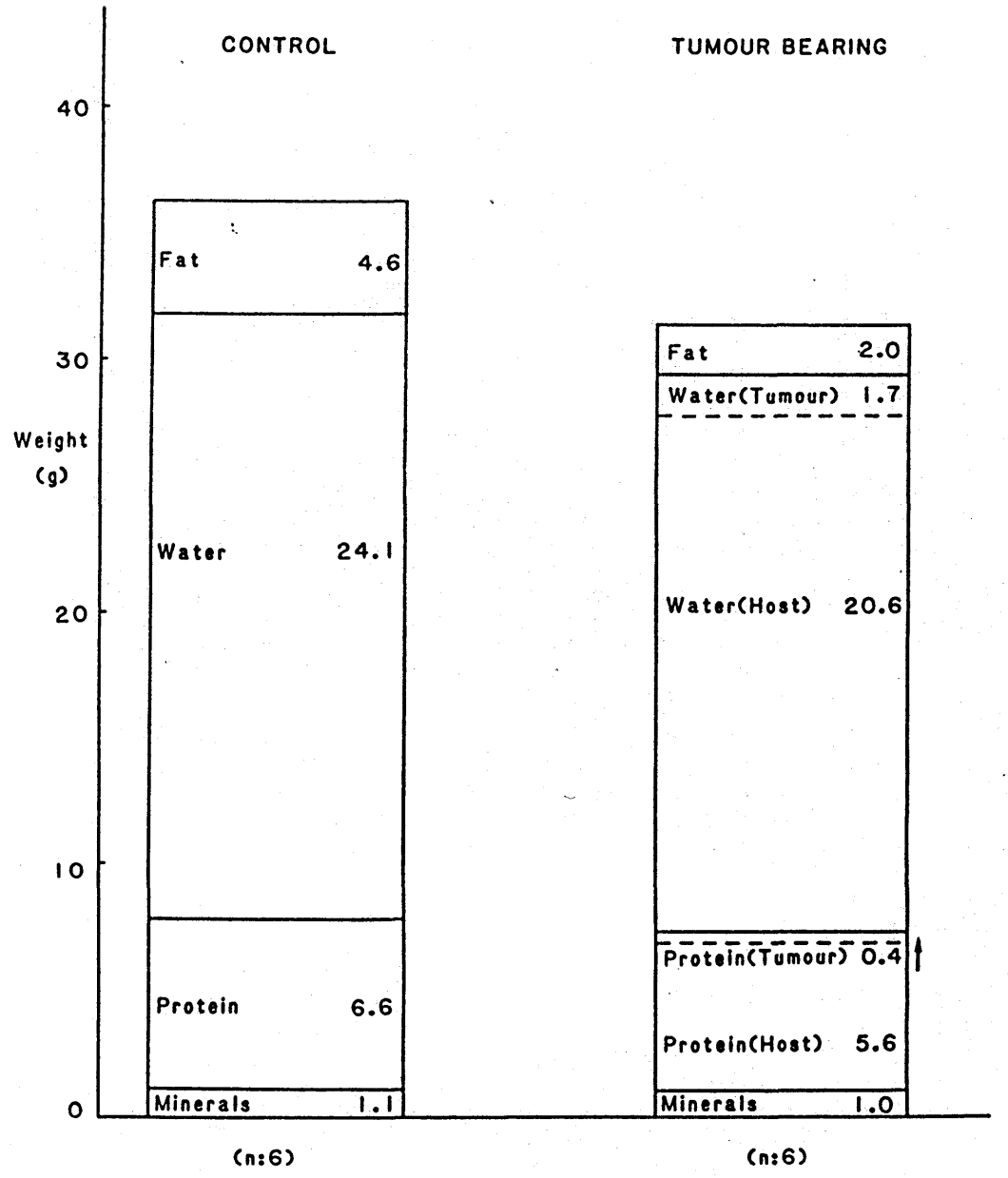


Figure 7.2 Changes in body composition of mice bearing the MAC-16 tumour. Two groups of six animals were matched for body weight. One group underwent tumour implantation and other sham operation. Twenty four days after tumour implantation both groups were sacrificed and body composition determined by neutron activation analysis.

However, total body protein was reduced by 9% (6.0 $\pm$ 0.1 cf 6.6 $\pm$ 0.3g : $P < 0.05$). Tumour protein (0.35 $\pm$ 0.05g) accounted for 5% of total body protein mass and total body fat was reduced by more than 55% (2.0 $\pm$ 0.2 cf 4.6 $\pm$ 0.4 : $P < 0.01$).

Discussion

The Walker 256 was a very poorly differentiated anaplastic tumour. There was no evidence of smooth muscle filaments, deposition of extracellular collagen or spindle cell formation. Therefore, the designation carcinosarcoma is probably invalid and, as previously suggested (Fisher and Fisher, 1960), the tumour should be regarded as an epithelial malignancy. In contrast, the MAC-16 appeared to be a well differentiated adenocarcinoma. The presence of junctional complexes confirmed the epithelial nature of the tumour. Furthermore, well developed mitochondria, a prominant Golgi apparatus and sublumenal membrane-bound vesicles confirmed the secterory nature of the cells.

The wasting associated with the early stages of growth of the Walker 256 tumour was almost entirely due to the transfer of nitrogen from the host to the tumour (Figure 7.1). This phenomenon has led previous investigators to suggest that the tumour acts as a 'nitrogen trap' for host amino acids (Mider, 1948). The mechanism whereby host protein is broken down to provide amino acids for tumour protein synthesis is unknown. However, in humans, cachexia can occur when the tumour is less than 1% of body weight (Morrison, 1976) and therefore 'nitrogen trapping' by the tumour is unlikely to be an important mechanism of host weight loss. In contrast, the growth of the MAC-16 tumour was associated with greater losses by the host than were gained by the tumour (Figure 7.2): a situation more in keeping with that

observed in human cancer cachexia (see Chapter 5). The mechanisms of weight loss in this model might give a valuable insight into that of human cancer cachexia. Therefore, the food intake and energy and protein metabolism of mice bearing the MAC-16 tumour was studied to try and elucidate the mechanism of such excess weight loss.

Part 2: Energy balance and protein synthesis rates of mice bearing the MAC-16 tumour.

Materials and Methods

Animals, diet and tumour: The MAC-16 tumour was implanted in male NMRI, mice as described on page 93. The diet of the animals was as described on page 92.

Estimation of daily Energy expenditure: Energy expenditure was determined by indirect calorimetry. Rates of oxygen consumption and carbon dioxide production were measured in an open circuit, ventilated metabolism cage connected to a sensitive paramagnetic oxygen analyser and an infra-red carbon dioxide analyser. The system is described in detail in Chapter 3.

Estimation of protein synthesis rates in vivo: Mice were injected via a lateral tail vein with 1ml of phenylalanine (150mmol) which contained L-[4-^{3}H]-phenylalalanine at a dose of 100uCi/100g body weight. Ten minutes later animals were anaesthetised with diethyl ether and the liver, heart, right kidney and right gastrocnemius muscle were excised and rapidly frozen in liquid nitrogen. The fractional rate of protein synthesis was calculated from the ratio of the specific radioactivities of free and protein-bound phenylalanine measured as

described by Garlick and coworkers (1980) and outlined in Chapter 2. Tissue protein contents were estimated as the total ninhydrin positive material obtained after acid hydrolysis.

Experimental design

Energy expenditure and food intake of mice with MAC-16 tumour: Mice (n = 8) were housed individually in wire bottomed, perspex metabolism cages equipped with food and water resevoirs. Body weight was measured daily for one week to ensure a stable food intake and body weight before calorimetry. On the eighth day food intake, oxygen consumption and carbon dioxide production were measured over the following 24 hours. Thereafter, tumours were implanted, the animals were maintained in the metabolism cages and on days 12, 19 and 26 measurements were repeated. Twenty seven days after tumour implantation animals were sacrificed and tumours excised and weighed.

Protein synthesis rates in tissues of mice with MAC-16 tumour: Mice were randomly assigned to two groups of 7 animals. One group underwent implantation of the MAC-16 tumour and the other sham operation. Twenty eight days later rates of protein synthesis rates were measured in the liver, kidney heart and gastrocnemius muscle of the animals. To compare the effect tumour growth with that of starvation a group of non-tumour bearing animals (n = 7) were deprived of food for 24 hours prior to measurement of protein synthesis.

Statistical analysis: Where appropriate, Student's T-test for paired or unpaired data was used to determine statistical significance.

Weight loss, food intake and energy expenditure of mice bearing MAC-16 tumour: The mean weights of mice before tumour implantation (day 0) and at 12, 19 and 26 days thereafter are shown in Figure 7.3. The weight of the mice was significantly reduced by the 12th day after tumour implantation (30.4 ± 0.4 cf 31.0 ± 0.5g : $P < 0.001$). Thereafter the weight of the mice decreased progressively until at 26 days it was reduced by about 20% (24.8 ± 0.4 cf 31.0 ± 0.5g : $P < 0.001$). At 27 days the mice were sacrificed. Tumour weight was 2.6 ± 0.4g which was 10% of total body weight.

The food intake of the mice before and at 12, 19 and 26 days after tumour implantation are shown in Figure 7.4. Prior to tumour implantation, the mean food intake of the mice was 4.6 ± 0.3g/mouse/day. This was not significantly altered until the fourth week of the study when the mean daily food intake was reduced by 13% (4.0 ± 0.3 cf 4.6 ± 0.3g; $P < 0.02$).

Rates of oxygen consumption, carbon dioxide production, the respiratory quotient and energy expenditure before tumour implantation and at 12, 19 and 26 days thereafter are shown in Table 7.1. Compared with the non tumour bearing state, oxygen consumption was the same throughout the period of tumour growth (e.g. day 0 cf day 26 : 153 ± 4 cf 152 ± 4 mmol O_2/mouse/24hr). There was a slight but not significant decrease in carbon dioxide production (145 ± 4 cf 150 ± 4 mmol CO_2/ mouse/24hr). Similarly, there was a trend towards a lower respiratory quotient (0.96 ± 0.01 cf 0.98 ± 0.01) but this difference was not statistically significant. There was no change in energy expenditure associated with implantation and growth of the MAC-16 tumour.

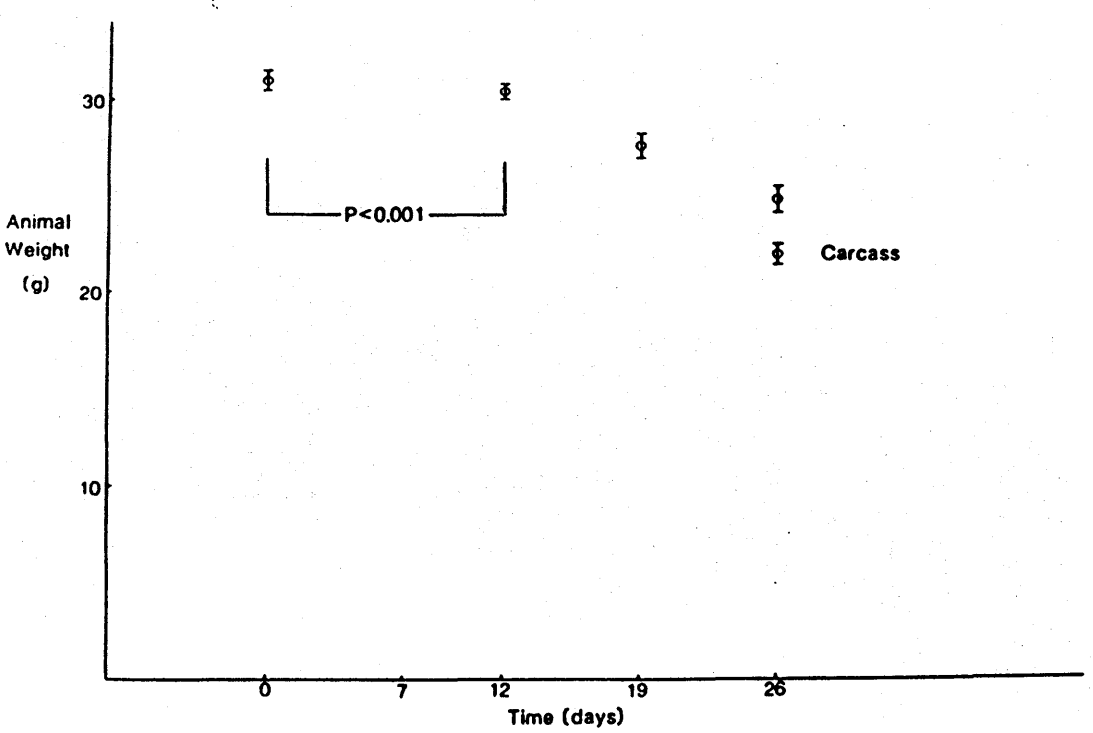


Figure 7.3 Effect of growth of MAC-16 tumour on body weight of male NMRI mice. Tumours were implanted on day 1. Animals were killed and tumours excised and weighed on day 26. Values are mean ± SEM.

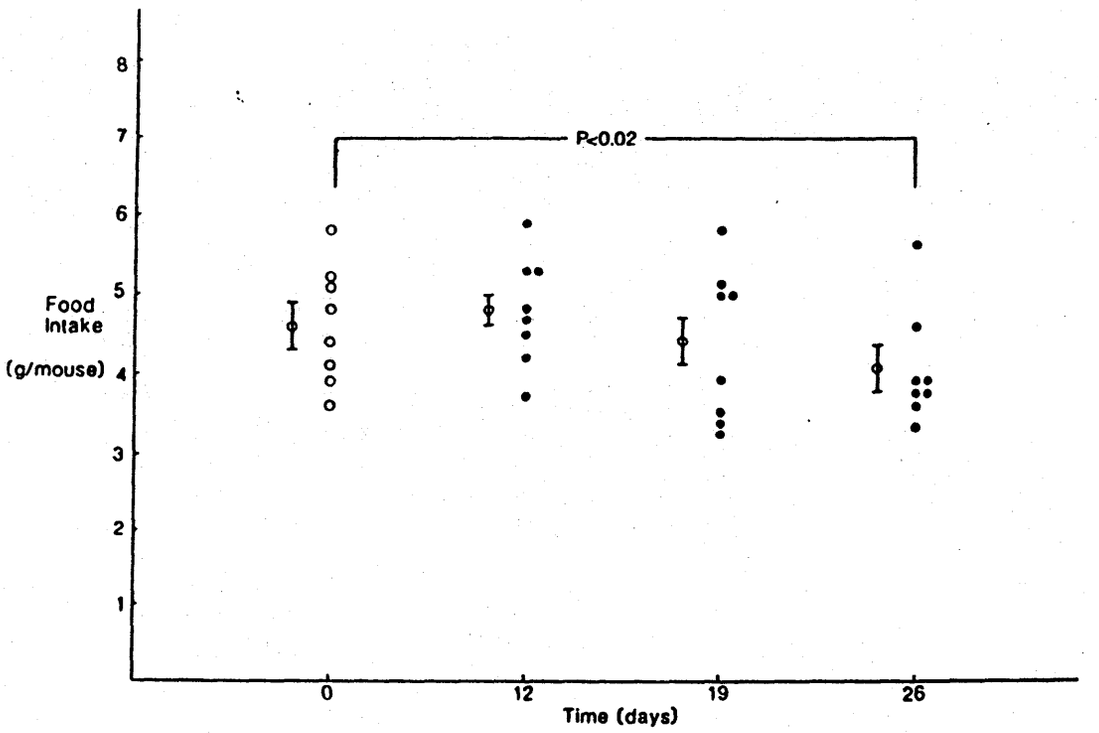


Figure 7.4 Effect of growth of MAC-16 tumour on daily food intake of male NMRI mice. Tumours were implanted on day 1. Values are mean ± SEM.

Time (days)	O_2 Consumption (mmol O_2/mouse/24h)	CO_2 Production (mmol CO_2/mouse/24h)	RQ[c]	Energy Expenditure (KJ/24h)
0[a]	153 (4)	150 (4)	0.98 (0.01)	65.9 (1.8)
12b	153 (4)	149 (3)	0.98 (0.02)	65.5 (1.3)
19	153 (6)	143 (4)	0.94 (0.02)	64.8 (2.4)
26	152 (4)	145 (4)	0.96 (0.01)	64.7 (1.4)

a, i.e. day before tmour implantation.

b, i.e. days after tumour implantation.

c, respiratory quotient

Table 7.1 Time course of O_2 consumption, CO_2 production and energy expenditure in mice before and after tumour implantation.
The MAC-16 tumour was implanted on day 1. Rates of O_2 consumption and CO_2 production were measured over 24hrs. Results are mean (s.e.m.) n = 8.

Rates of Protein synthesis in tissues of mice bearing the MAC-16 tumour: The fractional rate of protein synthesis in the liver, heart, skeletal muscle and kidney of normal and tumour bearing mice are shown in Table 7.2. There were no significant differences in rates of protein synthesis in the tissues of mice 28 days after tumour implantation compared with healthy controls. In contrast, after a 24h period of starvation rates of protein synthesis in non-tumour bearing mice were reduced in all tissues by 50%. The total protein content of the right gastrocnemius muscle was reduced by 33%, 28 days after implantation of the MAC-16 tumour (97.3 $\pm$ 9.6 cf 145.6 $\pm$ 12.1μM/muscle n = 7: $P < 0.01$).

Discussion

Mice bearing the MAC-16 progressively lost weight so that 18 days after tumour implantation body weight was reduced by 10% (Figure 7.3). During this period, neither food intake (Figure 7.4) or energy expenditure (Table 7.1) changed measurably. If host weight loss had been entirely due to loss of adipose tissue (the most energy rich tissue of the body) the total energy deficit would have been 87KJ which would have required a daily energy deficit of -4.8KJ/d ($\simeq$ 7% of energy intake or expenditure). Since the coefficient of variation of daily food intake was 16% and that of daily energy expenditure was 8% it is possible that a sufficient change in either variable to account for the daily energy deficit could have occurred and would not have been detected. This would concur with the conclusions reached from the human data (see Chapter 6). Alternatively, the efficiency of food digestion and intestinal absorption may have decreased so that the net energy available from food was reduced. Nevertheless, during the fourth week

	LIVER (%/day)	MUSCLE (%/day)	HEART (%/day)	KIDNEY (%/day)
NORMAL (fed ad lib)	182.2 (14.7)	32.2 (4.8)	28.7 (3.4)	85.7 (11.7)
TUMOUR BEARING (fed ad lib)	143.4 (23.5)	29.5 (4.5)	28.0 (2.5)	78.3 (9.0)
NORMAL (starved 24hrs)	70.5 (9.2)	16.6 (2.3)	11.9 (1.4)	35.8 (4.9)

Table 7.2 Fractional protein synthesis rate (%/day) in muscle, liver, kidney and heart of normal mice fed ad lib, normal mice starved for 24h and tumour bearing mice 28 days after implantation of MAC-16 and fed ad lib. Results are mean (s.e.m.), n = 7.

of tumour growth a negative energy balance was detected. This was due to a 13% reduction in food intake (Figure 7.4, $P < 0.02$) with no alteration in energy expenditure (Table 7.1).

These results contrast with the findings of other studies on the nature of the negative energy balance in cachectic tumour bearing mice. Lindmark and coworkers (1983) demonstrated a fall in energy expenditure but an even greater fall in food intake in weight losing mice bearing a methylcholanthrene-induced transplantable sarcoma. In contrast, Brooks and colleagues (1981) demonstrated a rise in energy expenditure with no change in food intake in cachectic immune deprived mice bearing a human hypernephroma. It is therefore apparent that the nature of the imbalance between energy intake and expenditure varies from one model of cancer cachexia to another. Moreover, since the nature of this imbalance in humans is unknown and may, indeed, vary from one individual to another it is impossible to determine which system should be used to investigate the aetiology of human cancer cachexia.

In the present study the food intake of the tumour bearing mice decreased but energy expenditure remained at a constant level. This contrasts with the normal adaptive response to a moderate reduction of food intake where body composition is maintained by a compensatory reduction in energy expenditure (Cahill, 1970; Lindmark et al., 1984). Protein synthesis is an a energy dependent process which is thought to account for 10 - 20% of energy expenditure in rodents and in man (Reeds, Fuller and Nicholson, 1985). Moreover, a fall in protein turnover is thought to be one of the main mechanisms for energy conservation during starvation (Waterlow, 1981). Twenty eight days after implantation of the MAC-16, rates of protein synthesis in the liver, kidneys, heart and skeletal muscle were the same in tumour

bearing animals as in controls (Table 7.2). In contrast, non-tumour-bearing mice fasted for 24h showed a 50% reduction in protein synthesis in all tissues (Table 7.2). It is therefore possible that the failure to reduce energy expenditure in response to weight loss and a reduced food intake was due to the absence of an adaptive fall in rates of protein synthesis (see also Chapter 6).

Two previous studies have determined rates of protein synthesis in the tissues of cachectic tumour bearing mice by the same method as in the current investigation. Emery and coworkers (1984b) observed that immune deprived mice bearing the XK1 human hypernephroma developed 20% weight loss when the tumour reached 5% of host body weight. During weight loss, food intake was reduced by about 15% and rates of protein synthesis in skeletal muscle and liver were reduced by 70% and 40% respectively. In contrast, Pain and coworkers (1984) reported rates of protein synthesis to be unaltered in skeletal muscle but increased in the liver of mice bearing the Ehrlich ascites tumour. In the present study no change in protein synthesis rates were observed following tumour growth and the development of cachexia. Therefore, it is apparent that host protein metabolism shows a heterogeneous pattern of response to the development of cancer cachexia. Furthermore, that increased rates of protein synthesis are the major cause of an increased energy expenditure and negative energy balance in tumour bearing mice is clearly incorrect since in XK1 tumour bearing mice energy expenditure is increased (Brooks et al., 1981) yet protein synthesis rates are markedly reduced (Emery et al., 1984).

The mechanisms of weight loss were investigated in two rodent models of cancer cachexia. In rats bearing the Walker 256 tumour host weight loss was entirely due to a transfer of protein and water from the host to the tumour. In contrast, mice bearing the MAC-16 adenocarcinoma sustained greater protein and fat losses than were gained by the tumour.

The energy and protein metabolism of mice bearing the MAC-16 tumour was studied to try and elucidate the mechanism of this excess weight loss. After 4 weeks of tumour growth a negative energy balance was detected due to a reduction in food intake with no change in energy expenditure. Rates of protein synthesis in the tissues of the mice were unchanged with tumour growth and weight loss. The failure of energy expenditure to adapt to a reduced food intake may have been due to the absence of an adaptive fall in rates of protein synthesis.

The pattern of changes in rates of food intake, energy expenditure and protein synthesis in mice bearing the MAC-16 tumour were different from those described in other murine models of cancer cachexia. This suggests a heterogenous mixture of metabolic abnormalities in cancer cachexia and may help to explain the difficulty in determining the causes of weight loss in humans with cancer cachexia.

CHAPTER 8

Effect of a host specific energy substrate on development of cachexia in tumour bearing rats

Introduction

As discussed previously (Chapter 1) more than 80% of hospitalised cancer patients show evidence of protein-energy undernutrition (Nixon et al., 1980) and cachexia is a major contributing factor to mortality in patients with malignant disease (Warren, 1932). Often, the side effects of ineffective antineoplastic therapy exacerbate the nutritional consequences of progressive tumour growth (Kokal, 1985). In an attempt to break this vicious circle, enteral and parenteral hyperalimentation have been extensively administered. The results of this approach have, however, been disappointing. Host reserves may only be partially restored (Nixon et al., 1981) and together with the dominance of progressive tumour growth this has meant that survival has not been prolonged (Brennan, 1981). This situation has encouraged the search for alternative methods with which to influence the host-tumour relationship.

One approach to this problem might be to maintain the host's energy supply in a form which cannot be used by the tumour. Tumour cells are known to have a high rate of glucose consumption, to show increased rates of anaerobic glycolysis, and to be susceptible to carbohydrate deprivation (see Demetrakopoulos & Brennan, 1982). Alternatively, many poorly differentiated tumours lack certain key mitochondrial enzymes and as a result have a greatly reduced ability to use fat or ketone bodies for energy production (Cederbaum & Rubin, 1976; Tisdale & Brennan, 1983).

Thus it has been proposed that ketone bodies might be administered as a "host specific" energy substrate (Tisdale & Brennan, 1983).

During starvation mobilization of free fatty acids from adipose tissue provides a source of energy for organs such as muscle or liver. Excess free fatty acids are converted to ketone bodies (acetoacetate and 3-hydroxybutyrate) in the liver and these serve as a source of energy for extrahepatic tissues including the brain (Owen et al., 1969). This leads to a decrease in overall glucose requirement and hence a minimal requirement for gluconeogenesis from amino acids provided by breakdown of protein in skeletal muscle.

In contrast to starvation, humans with malignant disease demonstrate increased rates of gluconeogenesis (Waterhouse, Jean Petre and Keilson, 1979; Holroyde et al., 1975). This phenomenon may allow for continued tumour growth in the wasted host, and might account for accelerated weight loss in tumour bearing individuals (Gold, 1971). However, dietary induced systemic ketosis has been shown to reduce blood glucose concentration and glucose utilisation in man (Phinney et al., 1983). Moreover, the supply of glycerol and alanine as precursors for gluconeogenesis may be decreased in the ketotic state (see Robinson & Williamson, 1980). Thus, iatrogenic ketosis has been proposed as a method of regulating host metabolism such that host reserves are maintained and glucose supply to the tumour is reduced (Conyers et al., 1979; Williams & Matthaei, 1981). A ketogenic diet has already been shown to reduce the growth rate of the tumour whilst maintaining that of the host in rats bearing a transplantable mammary cancer (Buzby et al., 1980).

However, the feasibility of this approach in humans has not been tested. Indeed, it has been suggested that hepatic ketogenesis may be impaired in cachectic cancer patients (Conyers et al., 1979). Thus it might be difficult to induce a state of ketosis in order to examine its effects on tumour growth rate and host metabolism. It is also not established whether ketone bodies have a direct inhibitory effect on muscle proteolysis. Thus it is unknown whether ketosis would reduce muscle wasting or decrease the supply of amino acids for gluconeogenesis. Skeletal muscle is thought to account for approximately 50% of protein turnover in the whole body (Emery et al., 1984a). Therefore, one indirect method of assessing the effects of ketosis on host muscle protein metabolism would be to determine the effects of ketosis on total body nitrogen turnover and balance.

The ethical and practical considerations of keeping terminal cachectic cancer patients in hospital for a prolonged period of time precluded the elevation of ketosis as an inhibitor of tumour growth rate in humans. Therefore, this question was examined using an animal model of cancer cachexia. The Walker 256 tumour in rats grows reproducably enough to make this assessment possible with a relatively small number of animals. Moreover, the use of this animal model allows sequential body composition analysis to assess the effect of ketosis on long term whole body nitrogen balance.

The aim of the present study was to determine the effects of systemic ketosis on tumour growth rate, host body composition changes and blood glucose concentration in rats bearing the Walker 256 carcinosarcoma. In addition, the activities of the three major enzymes responsible for the metabolism of ketone bodies were measured as an indication of the capacity of the tumour to utilize ketone bodies.

Animals: Inbred female Wistar rats aged between 12 - 14 weeks and weighing 175-205g were housed individually. They were kept in conditions of controlled temperature and lighting ($20^{o}C \pm 2^{o}C$, 12/12h light/dark cycle) and allowed free access to food (except where specified in text) and water.

Tumour: The rapidly growing Walker 256 carcinosarcoma was obtained from the Institute of Cancer Research, Sutton, Surrey and maintained by routine passage every 14 days in Wistar rats. Viable tumour fragments (100mg) were transplanted s.c. into the right flank under aseptic conditions and light ether anaesthesia. Tumour doubling time was about 36h.

Diets:

Standard diet: The standard diet was CRM diet (Labsure, Rank-Hovis-McDougal, Agricultural Division, Dorset, U.K.). It contained 18.5% protein, 2.5% fat, 56.0% carbohydrate, 4.3% fibre, 2.4% minerals, 15.0% moisture and added vitamins and trace elements.

Ketogenic diet: Medium chain triglyceride (MCT, donated by Scientific Hospital Supplies Ltd., Liverpool, U.K.) was mixed with the standard diet and supplemented with protein powder (Maxipro: Scientific Hospital Supplies) so that the two diets contained the same quantity of protein per calorie. Vitamin supplements were also added. The MCT supplied 70% of the total calories and this diet is referred to as the MCT diet.

Both diets were presented to the animals as a paste to minimise food scatter.

Neutron activation analysis: Total body nitrogen was analysed by neutron activation analysis as described in Chapter 4.

Determination of tumour enzyme activities: The activities of 3-hydroxybutyrate dehydrogenase, acetyl CoA acetyltransferase and 3-ketoacid CoA transferase were determined spectrophotometrically as described by Tisdale & Brennan (1983). The tumour was surgically excised from the rat 8 days after implantation and homogenised at $4^{o}C$ in 4 vol of ice cold Tris-HCL buffer, $10^{-2}M$ pH 7.4, containing 2-mercaptoethanol $10^{-3}M$ and sucrose 0.25M. The homogenate was then further dispersed by ultrasonic vibration for 30 sec. The homogenate was centrifuged at 30,000g for 20 min and the supernatant used for enzyme estimation. Activity, estimated in triplicate, is expressed as units/mg protein where one unit equals the amount of substrate converted in 1 min at $37^{o}C$.

Induction of ketosis: The Walker 256 carcinosarcoma was transplanted into 20 rats which were fed ad libitum on the MCT diet. Concurrently, another group of 20 tumour free rats were fed ad libitum on the MCT diet. After either 3, 6, 9, 12 or 15 days four animals from each group were killed and blood was taken for the estimation of the concentration of acetoacetate and 3-hydroxybutyrate.

Effects of ketosis on tumour weight and host body composition: Rats were randomly assigned to four groups of 6 animals. Animals were fed on the paste diets, contained in glass beakers, for a period of 7 days. Food intakes were measured daily and all animals had attained a stable food intake by the seventh day. On the eighth day, total body nitrogen was determined by neutron activation analysis in vivo and this was followed by either tumour implantation (Groups A, B, C) or sham

operation (Group D). Animals in Group A were subsequently fed ad libitum on the standard diet and those in Group B were fed ad libitum on the MCT diet. Since animals fed on the MCT diet ate slightly less than those on the standard diet, Groups C and D were included to act as controls for the reduced dietary intake. Each animal in Group C was given a daily ration of the standard diet equivalent in energy and nitrogen content, to that consumed on the previous day by its partner animal in Group B. The tumour free animals (Group D) were fed on the MCT diet and were similarly pair fed to Group B.

After 14 days all animals were killed and blood was taken for measurement of 3-hydroxybutyrate, acetoacetate and glucose concentrations. The tumours were then excised and weighed. Neutron activation analysis of both carcasses and tumours was then repeated.

Analysis of rat blood samples: Acetoacetate and 3-hydroxybutyrate were measured spectrophotometrically by the method of Williamson and Mellanby (1974). Glucose was measured spectrophotometrically by the method of Berger and Bernt (1974).

Statistical analysis: Students t test for non-paired data was used.

Results

Enzyme activities estimated in the Walker 256 tumour: Table 8.1 shows the activities of the three major enzymes responsible for the metabolism of ketone bodies, estimated in an homogenate of the Walker 256 tumour. Significant activities of 3 hydroxybutyrate dehydrogenase and acetyl CoA acetyl-transferase were observed. However, no activity of the enzyme 3-ketoacid CoA transferase could be detected in the tumour homogenate (Table 8.1).

Enzyme	Enzyme activity units/mg protein
3-Hydroxybutyrate dehydrogenase	4.8
Acetyl CoA acetyltransferase	12.5
3-Ketoacid CoA transferase	Nil

TABLE 8.1 Activity of 3-hydroxybutyrate dehydrogenase, acetyl CoA acetyltransferase and 3-ketoacid CoA transferase in Walker 256 carcinosarcoma. Values represent the mean of at least three observations. Units of enzyme activity are as defined in **Materials and Methods**

Effects of the MCT diet on blood ketone body levels: Figure 8.1 shows the total ketone body concentration in blood taken from rats fed ad libitum for various times on the MCT diet (open bars). A significant ketosis was observed in rats fed for only 3 days on the MCT diet and this level was maintained for at least 15 days. Also shown in Figure 8.1 is the total ketone body concentration in blood taken from rats fed ad libitum on the MCT diet for various times after implantation of the Walker 256 tumour (hatched bars). The level of ketosis observed in the tumour-bearing rats was similar to that observed in the non-tumour-bearing rats.

Effects of the MCT diet on tumour growth rate and host body weight: Host body weight before (hatched bar) and 14 days after tumour implantation into rats fed ad libitum on the standard diet (stippled bars, Group A) is shown in Figure 8.2. There was a significant decrease (by 12.0%) in the body weight of the rats by the 14th day after tumour implantation ($P < 0.01$).

A similar decrease in body weight (by 14.6%) was observed in tumour bearing rats fed ad libitum on the MCT diet (Figure 8.2 Group B). The daily energy intake of the tumour-bearing rats fed ad libitum on the MCT diet (38.2KJ/rat/day) was less than that of the tumour-bearing rats fed on the standard diet (43.3KJ/rat/day). When tumour bearing rats, fed on the standard diet, were pair fed to the tumour-bearing rats fed ad libitum on the MCT diet (Group B), such that the mean intake of standard diet was 38.2KJ/rat/day, there was a slight, but not significant ($P > 0.05$), increase in the host body weight loss (Figure 8.2, Group C cf Group A). Non-tumour-bearing rats restricted to a mean daily intake of 38.2KJ/rat/day of the MCT diet also lost weight after 14 days of this dietary regime (Figure 2, Group D). The weight loss of animals in Group D (12.1%) was similar to that of tumour bearing rats fed ad libitum on the MCT diet (Group B).

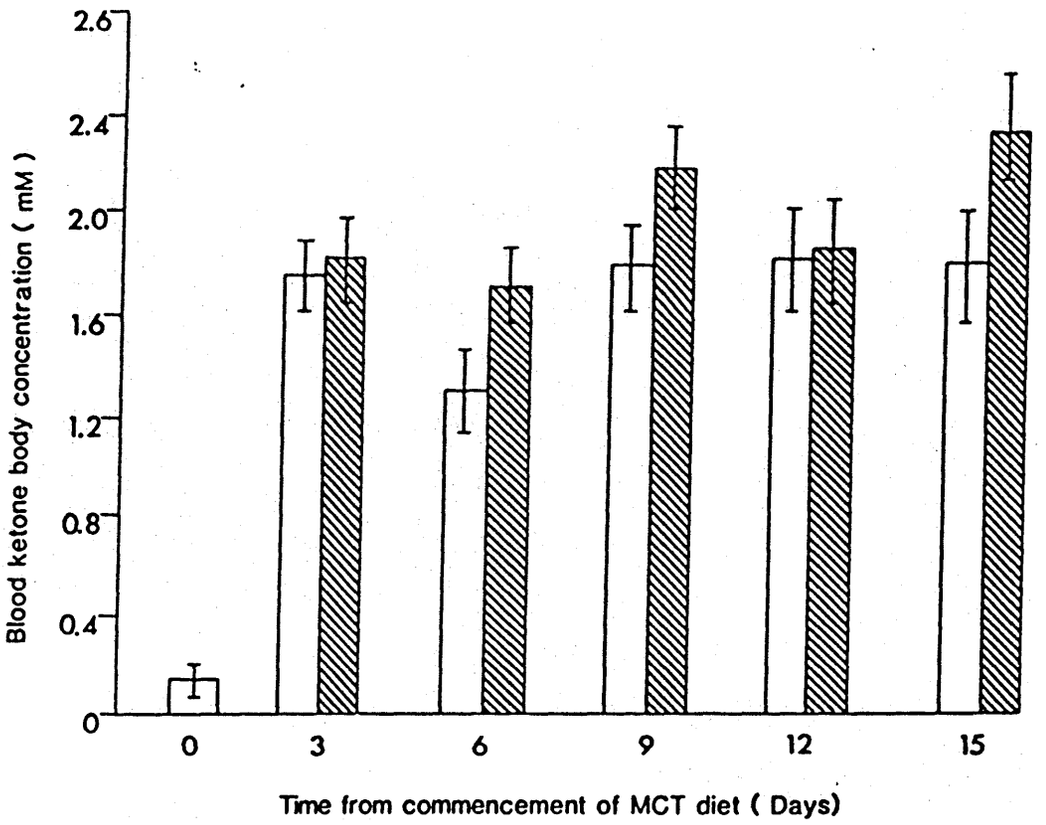


Figure 8.1 Ketone body concentration estimated in blood from rats fed for various times on a 70% medium chain triglyceride diet. Rats were either non-tumour-bearing (open bars) or underwent tumour implantation on day 0 (hatched bars). Values are mean ± SEM (n = 4).

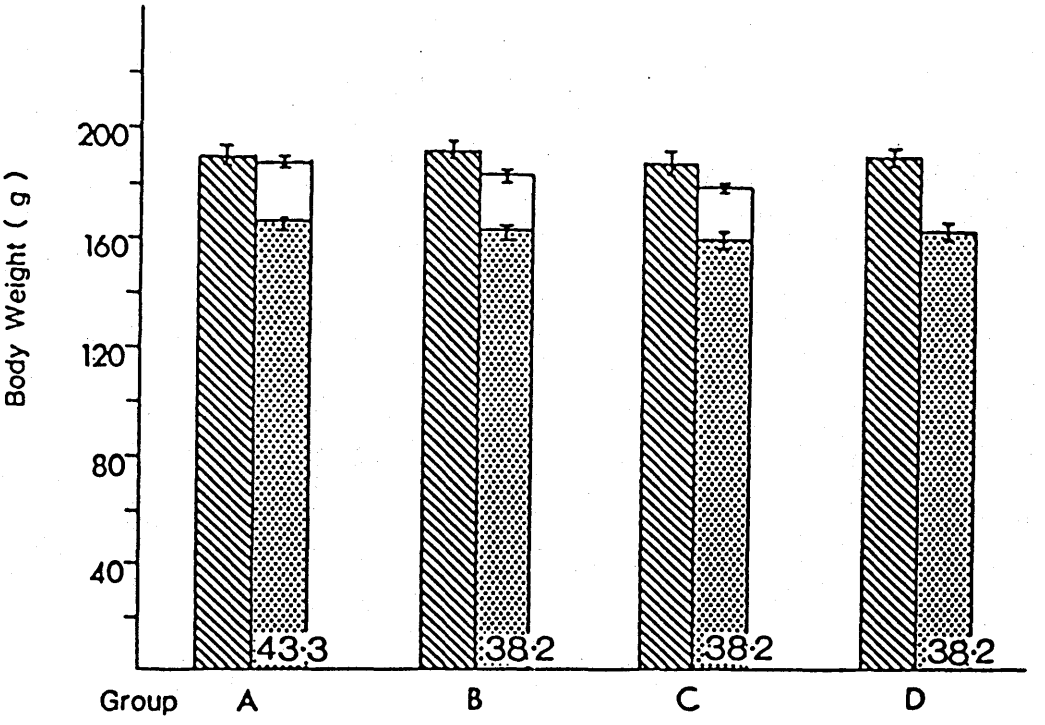


Figure 8.2 Effects of systemic ketosis on host weight loss following growth of the Walker 256 tumour in rats. Body weight of rats before (hatched bars) and fourteen days after tumour implantation (stippled bars) is shown. Final tumour weight is also shown (open bars). Tumour bearing animals were fed either on the standard diet ad libitum (Group A) or on the MCT diet ad libitium (Group B). Animals in Group C were pair fed with standard diet to those in Group B. Animals in Group D were non tumour bearing and pair fed with MCT diet to those in Group B. Values are mean ± SEM (n = 6). Mean daily energy intake of the animals is shown within the bars (Values are KJ/rat/day).

The final tumour weight was similar for all three dietary regimes (Figure 8.2, open bars).

Effect of the MCT diet on the body composition of tumour-bearing rats: Figure 8.3 shows the total nitrogen content of rats both before (hatched bars) and 14 days after (stippled bars) tumour implantation. The final nitrogen content of the tumour is also shown (open bars). The rats were fed ad libitum on either the standard diet (group A) or the MCT diet (Group B). After 14 days the total nitrogen content of the rats had decreased and the decrease was similar for rats fed ad libitum on either the standard diet (0.63gN, Group A) or the MCT diet (0.78gN, Group B). The final nitrogen content of the tumour was slightly greater in rats fed ad libitum on the standard diet (0.55gN) than in rats fed ad libitum on the MCT diet (0.46gN) but this difference was not significant ($P > 0.05$). When the daily intake of the standard diet was restricted to that of the tumour-bearing rats fed ad libitum on the MCT diet (Group B) the final host nitrogen content and tumour nitrogen content was similar to that of the tumour bearing rats fed ad libitum on the MCT diet (Figure 8.3, Group C cf Group B). When non-tumour bearing rats were fed for 14 days on the MCT diet but restricted to a mean daily energy intake of 38.2KJ there was a slight, but not significant ($P > 0.05$) decrease in the body nitrogen content (Figure 8.3, Group D).

Blood glucose and ketone body concentrations of tumour-bearing rats on the various dietary regimens: Table 8.2 shows the final blood glucose concentration and total ketone body concentration of rats fed ad libitum for 14 days on either the standard diet (group A) or the MCT diet (group B). A significant ketosis was observed in tumour-bearing rats fed ad libitum for 14 days on the MCT diet and this was

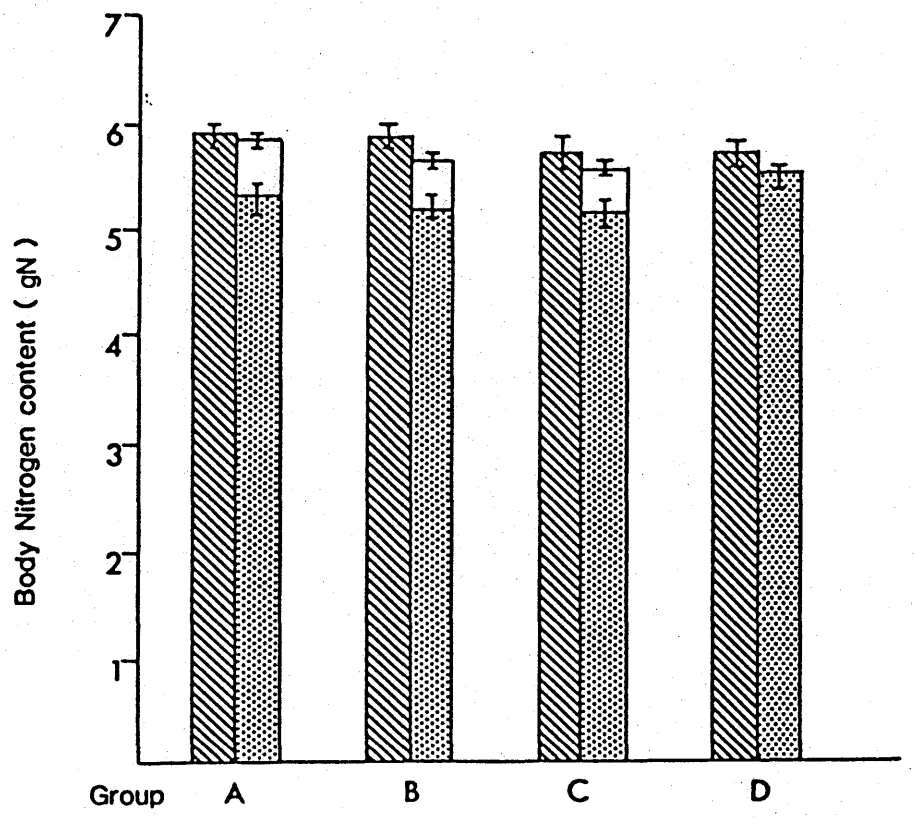


Figure 8.3 Effect of systemic ketosis on host body nitrogen following growth of the Walker 256 tumour in rats. Body nitrogen content of rats before (hatched bars) and after tumour growth (stippled bars) is shown. Final tumour nitrogen content is also shown (open bars). Animal groups were as defined in Figure 8.2. Values are mean ± SEM (n = 6).

Group	Total Blood Ketones (mM)	Blood Glucose (mM)
Control	0.14 ± 0.07	6.5 ± 0.3
A	0.25 ± 0.09	6.2 ± 0.3
B	2.68 ± 0.59	5.2 ± 0.4
C	0.29 ± 0.08	5.3 ± 0.2
D	2.10 ± 0.22	3.4 ± 0.4

Table 8.2 Total ketone body concentration (acetoacetate plus 3-hydroxybutyrate) and glucose concentration in blood from rats 14 days after tumour implantation (Groups A, B and C) and in non-tumour-bearing rats (Group D). Control values refer to non-tumour-bearing rats fed ad libitum on the standard diet. Animal groups were as defined in Figure 8.2. Values are mean ± SEM (n = 6).

accompanied by a significant ($P < 0.02$) reduction in the blood glucose concentration. When the daily energy intake of non-tumour-bearing rats on the MCT diet was restricted to that of the tumour-bearing rats fed on the MCT diet the blood ketone concentration was similar but the blood glucose concentration was significantly reduced (Table 8.2 Group D cf Group B: $P < 0.01$).

Discusssion

These results demonstrate clearly that induction of systemic ketosis following implantation of the Walker 256 carcinosarcoma in rats does not reduce the tumour growth rate, nor does it prevent the subsequent decrease in host body weight. Furthermore, sequential body composition analysis of the rats indicated that the host nitrogen content decreased to a similar extent in tumour bearing animals fed on the MCT diet compared with those fed on the standard diet (Figure 8.3).

Two of the major enzymes responsible for the metabolism of ketone bodies in the mitochondria, 3-hydroxybutyrate dehydrogenase and acetyl CoA acetyltransferase, were shown to be present in significant amounts in the Walker 256 tumour (Table 8.1). However, no activity of the enzyme 3-ketoacid CoA transferase was detected. A similar distribution of enzyme activities has been reported for other tumours of the peripheral tissues (Tisdale and Brennan, 1983). Since activity of the enzyme 3-ketoacid CoA transferase determines the extent to which 3-hydroxybutyrate is used as a metabolic fuel (Williamson et al., 1971) it might be expected that the Walker 256 carcinosarcoma would be unable to metabolize ketone bodies. However, it has been shown previously that Walker 256 tumour cells grown in vitro can form $^{14}CO_2$ from D(-)3-hydroxy 3-^{14}C-butyrate, but only at a very low rate due to the

presence of acetoacetyl CoA synthetase (Tisdale, 1984). Thus, although the tumour has a limited capacity for the metabolism of acetoacetate the low activity of acetoacetyl CoA synthetase and the absence of 3-ketoacid CoA transferase indicates that ketone bodies cannot be a major energy source for this tumour.

The diet chosen for use in these studies produced a significant systemic ketosis when fed to rats for only 3 days; this ketosis was maintained in rats fed on the MCT diet for at least 15 days (Figure 8.1). When non-tumour-bearing rats were fed on the MCT diet the increased concentration of ketone bodies in the blood was accompanied by a significant decrease ($P < 0.01$) in the blood glucose concentration (Table 8.2). This is consistent with the observation that ketosis induced in humans by a diet containing 85% of calories as fat is associated with signficant reduction in the blood glucose concentration (Phinney et al., 1983). Similarly, the induction of a systemic ketosis by the infusion of 3-hydroxybutyrate into man (Sherwin et al., 1975), dogs (Binkiewicz et al., 1974) or sheep (Radcliffe et al., 1983) has been shown to produce a significant decrease in the blood glucsoe concentration and is associated with a decrease in the rate of gluconeogenesis of both fed and fasted sheep (Radcliffe et al., 1983). However, although a similar degree of ketosis was induced in both tumour bearing and non-tumour-bearing rats fed on the MCT diet (Figure 8.1) the decrease in the blood glucose concentration of the tumour-bearing rats, though significant, was much less marked (Table 8.1). Furthermore, the limited decrease in blood glucose concentration of tumour-bearing rats fed ad libitum on the MCT diet could be accounted for by the decrease in their daily calorie intake since tumour-bearing rats fed an equicaloric amount of the standard diet had a similar blood glucose concentration (Table 8.2).

In septic sheep systemic ketosis is not accompanied either by a decrease in the blood glucose concentration or by a decrease in the rate of glucose production by the liver (Radcliffe et al., 1983). This observation lends support to the suggestion that the metabolic abnormalities observed in the tumour-bearing host resemble some of those characteristic of semi-starvation but also some of those characteristic of sepsis and trauma (Brennan, 1977). The mechanism accounting for the failure of systemic ketosis to lower blood glucose in sepsis or cancer cachexia remains obscure. It may, however, be related to the insulin resistance characteristic of both metabolic states.

The failure of the ketogenic diet to restrict tumour growth may be, therefore, a result of the failure of ketosis to reduce the availability of glucose in tumour-bearing rats. It has already been shown that the growth rate of the Walker 256 tumour in rats can be decreased following administration of the glucose antimetabolite 2-deoxy-glucose (Ball et al., 1957) or following administration of hydrazine sulphate which inhibits gluconeogenesis (Gold, 1971). This suggests that the Walker 256 tumour is largely dependent upon glucose as an energy substrate. Thus, although the tumour cells are probably unable to use ketone bodies as a major energy source, the ketogenic diet did not restrict the supply of glucose to the tumour cells and thus failed to deprive the tumour of an important energy source.

The final tumour weight in rats fed ad libitum on the MCT diet was slightly, but not significantly ($P > 0.05$) less than that in rats fed ad libitum on the standard diet (Figure 8.2, Group B cf Group A). The slight decrease in tumour growth rate can be accounted for entirely by the reduced energy intake since a similar decrease in the final tumour size was observed in rats that had a daily allowance of the standard

diet equal in energy content to that eaten by rats fed on the MCT diet (Figure 8.2, Group C cf Group B).

Although it cannot be concluded from this study that a host specific substrate does not restrict tumour growth, the results indicate the difficulty of such an approach to cancer treatment. The presence of metabolic abnormalities in cancer-bearing patients has been widely documented (Brennan, 1977; Heber, Byerly and Chlebowski, 1985) and these abnormalities may alter the host's response to dietary modification. The effect of a ketogenic regimen on the metabolism of humans with cancer cachexia is described in Chapter 9.

Summary and Conclusions

The Walker 256 carcinosarcoma was shown to lack the enzyme 3-Keto Acid CoA transferase. This suggests that ketone bodies cannot be used as a major substrate for the energy metabolism of this tumour. Systemic ketosis (1 - 2mM acetoacetate plus 3-hydroxybutyrate) was induced both in tumour bearing and in non tumour bearing rats with a diet containing 70% medium chain triglyceride. However, in rats bearing the Walker 256 tumour, this dietary ketosis did not reduce the tumour growth rate nor did it prevent the subsequent decrease in host body weight. Host body nitrogen losses were similarly unaffected. The ketosis induced in tumour bearing rats was shown to be abnormal since the blood glucose concentration of ketotic tumour bearing rats was significantly higher compared with that of ketotic non-tumour bearing rats (5.2 ± 0.4mM cf 3.4 ± 0.6mM, $P < 0.01$). The failure of glucose metabolism to respond normally to ketosis may partly explain why systemic ketosis failed to alter the growth and cachexia induced by the Walker 256 tumour.

CHAPTER 9

Influence of systemic ketosis on substrate levels and nitrogen metabolism in humans with cancer cachexia

Introduction

It was concluded in Chapter 8 that induction of systemic ketosis in tumour-bearing rats does not reduce the tumour growth rate nor does it prevent host weight loss. The study was based on the assumption that the presence of ketone bodies would limit the supply of glucose to the tumour and thus restrict its growth rate. Since the host tissues can use ketone bodies as a substrate their energy metabolism should not be affected. However, in rats at least, induction of systemic ketosis did not restrict the supply of glucose to the tumour. This may be an indication of one of the many metabolic abnormalities induced in the host by the presence of a tumour. However, it may also be a characteristic of the particular animal tumour model chosen for use in the study. Certainly induction of systemic ketosis in humans by a high fat diet results in a significant reduction in the blood glucose concentration (Phinney et al, 1983) as does infusion of 3-hydroxybutyrate (Sherwin et al., 1975).

One mechanism for the hypoglycaemic action of systemic ketosis in unstressed humans or rats (Phinney et al., 1983; Chapter 8) might be that ketone bodies directly inhibit protein breakdown in skeletal muscle. Thus the efflux of amino acids from skeletal muscle would be reduced and since gluconeogenesis is to some extent controlled by substrate availability (Newsholme, 1976) this would reduce the rate of glucose synthesis *de novo*. However, as discussed in Chapter 8, ketone bodies may have no direct action on muscle proteolysis (Miles et al., 1983). Although urinary nitrogen excretion falls after the intravenous

infusion of sodium 3-hydroxybutyrate (Sherwin, Hendler and Felig, 1975 ; Pawan and Semple, 1983) this effect can be reproduced by an equimolar infusion of bicarbonate (Miles and Haymond, 1983 ; Bartels and Sestoft, 1983). Since the metabolism of salts of organic acids, such as 3-hydroxybutyrate, results in an equimolar generation of bicarbonate ions, the administration of such salts probably reduces urinary nitrogen by decreasing the need for ammonium ion excretion by the kidney rather than by direct inhibition of muscle proteolysis (Bartels and Sestoft, 1983). If ketone bodies do not reduce muscle proteolysis this might explain why host nitrogen losses were not reduced in tumour bearing rats fed a ketogenic diet (see Chapter 8).

It has also been suggested that cancer patients exposed to a ketogenic regimen may not achieve the same level of ketosis as observed in normal individuals (Conyers et al., 1979). Thus a standard ketogenic diet in which endogenous ketogenesis is induced by carbohydrate restriction might not increase circulating ketone body concentrations to the extent that the metabolic effects of systemic ketosis in cancer cachexia could be determined. One approach to this problem would be to administer an exogenous source of ketone bodies at the same time as inducing hepatic ketogenesis by carbohydrate restriction. This type of diet was used in the current study.

Thus the aim of this study was to determine whether an adequate ketosis could be induced in cachectic cancer patients and if so, to determine the effects of this ketosis on the nitrogen metabolism, blood glucose concentration, and the concentration of various substrates of hepatic gluconeogenesis in these patients. Systemic ketosis was induced by feeding the patients a diet containing 70% medium chain triglyceride supplemented with arginine 3-hydroxybutyrate, 4mmol/Kg/day. The

arginine salt was chosen in order to avoid the fluid retention which might be associated with a sodium load of 4mmol/Kg/d. Patients were fed on a normal diet for six days and this was followed by seven days on the ketogenic diet. Nitrogen retention has been shown to vary with energy intake (Elia, 1982). In general, increasing energy intake improves nitrogen balance at any given nitrogen input. Similarly, a rise in nitrogen input will improve nitrogen retention at any given energy intke. Therefore, in order to examine the effect of ketosis on nitrogen metabolism both diets were carefully balanced to be iso-nitrogenous and isocaloric. Blood samples were taken for estimation of blood glucose concentration and the concentration of various substrates of gluconeogenesis when the patients were on either regimen. Moreover, to determine the effect of systemic ketosis on host nitrogen metabolism daily nitrogen balance was measured throughout, and whole body protein turnover estimated on the last day of both the normal and ketogenic diets. The effect of ketosis on tumour growth rate was not assessed in this study.

Materials and Methods

Subjects: 5 patients, two male and three female with histologically proven malignancy were entered into the study. Two had lung cancer, two gastric cancer and one ovarian cancer. Details of each patient's weight, diagnosis, age, sex, height, weight and % weight loss are given in Table 9.1. Details of the patients' nutritional and haematological status at the start of the study are given in Table 9.2. Twenty four hour dietary recall histories obtained prior to the start of naso-gastric feeding showed the mean daily energy intake of the patients to be 4.6MJ/d and the mean daily nitrogen intake to be 7.7gN/d. These values are 55% and 28% respectively below predicted normal intakes

Patient	Diagnosis	Age (yr)	Sex	Height (cm)	Weight (kg)	Pre-illness weight (kg)	%Weight loss	Duration of weight loss (m)	Weight loss per month (kg)
1	Ovary	64	F	155	32.0	46.0	30	10	1.4
2	Lung	59	M	168	45.0	63.5	29	12	1.5
3	Gastric	73	F	152	35.0	48.9	28	8	1.7
4	Lung	58	F	155	31.0	51.0	39	10	2.0
5	Colon	52	M	171	50.0	73.0	32	10	2.3
mean	-	61	-	160	38.8	56.5	32	10	1.8
(sem)	-	(4)	-	(4)	(3.8)	(51)	(2)	(0.4)	(0.2)

TABLE 9.1 Characteristics of patients undergoing nasogastric feeding.

Patient	Albumin (g/l)	Trans-ferrin (g/l)	24hr Urinary Creat-inine (mmol/d)	Lymphocyte count ($x10^9/l$)	Hb (g/dl)	White Cell Count ($x10^9/l$)	Platelet count ($x10^9/l$)
1	38	0.9	1.4	0.4	11.8	6.7	335
2	31	1.6	4.6	0.4	12.6	4.7	345
3	25	1.7	3.7	0.8	9.2	4.1	242
4	30	0.9	2.8	0.9	11.1	8.8	401
5	31	1.3	6.5	0.9	12.3	7.9	392
mean (sem)	31 (2)	1.3 (0.2)	3.8 (0.8)	0.7 (0.1)	11.4 (0.6)	6.4 (0.9)	343 (28)

TABLE 9.2 Nutritional status and haematological indices of patients before start of nasogastric feeding.

(Documenta Geigy, 1962). All patients gave informed, written, consent. The study was approved by the local hospital ethical committee.

Diets: For the first six days of the study, the subjects were given a normal balanced regimen consisting of a lactose free commercial diet (Ensure, Ross Laboratories, USA). The diet provided 44 Kcal/kg/d and 1.5 g protein/kg/d and was supplemented with arginine 4mmol/kg/d. In the normal diet 55% of the total energy was supplied as carbohydrate and 31% as fat. From the seventh day of the study onwards, the patients were fed a ketogenic diet which supplied the same total energy and protein as the normal diet but in which 70% of the calories were supplied as medium chain triglyceride (Liquigen, Scientific Hospital Supplies Ltd, Liverpool, U.K.). This diet was supplemented with arginine D-3-hydroxybutyrate 4mmol/kg/d (a gift from Solvay Ltd, Brussels, Belgium). The protein source in the ketogenic was supplemented whey protein (Maxipro HBV, Scientific Hospital Supplies).

Both diets were administered at a constant rate over 24 hours using a fine bore nasogastric feeding tube connected to a peristaltic pump. The diets were well tolerated by the patients and there was no gastrointestinal upset.

Study Protocol: Patients received the normal diet on days 1 - 6 and the ketogenic diet on days 7 - 13. Twenty four hour urine collections for estimation of nitrogen balance were taken throughout the study. Blood samples for the estimation of urea and electrolytes were taken at 9 am on days 0, 5, 8, 10 and 13. Blood samples for the estimation of substrate concentrations were taken at 9 am on days 5, 8, 10, 13 and for insulin concentrations on days 5 and 13. Whole body protein turnover was measured on days 5-6 and 12-13.

Analysis of blood and urine samples: Blood for haemoglobin,white blood cell and differential counts, platelet counts, urea and electrolytes, and liver funtion tests was analysed by semiautomated techniques on the same day as sampling.Blood taken for 3-hydroxybutyrate, aceto-acetate, and pyruvate was immediately deproteinised with 30% ice cold perchloric acid and analysed within 3 hours of sampling. Plasma was separated from all other blood samples and frozen at -20^{o}C until analysis. The glucose, lactate, and pyruvate assays were enzymatic determinations taken from the standard techniques of Bergmeyer (1971).
3-Hydroxybutyrate and acetoacetate were measured by the method of Williamson and Mellanby (1974). Free fatty acids were measured enzymatically (NEFAC kit, Wako Chemicals, West Germany). The amino acid profiles were determined by ion exchange HPLC on plasma which had been deproteinised with sulphasalicylic acid. Insulin concentration was measured by radioimmunoassay.

Serial 24-hour urine collections were stored in bottles at 4^{o}C during the collection period. The volume was carefully measured after completion and aliquots stored at -20^{o}C until analysis. Total urinary nitrogen was measured by the micro-Kjeldahl technique (Fleck and Munro, 1965). Urinary creatinine was measured according to the modified method of Folin-Wu (Hawk, Oser and Summerson, 1947).

Measurement of protein turnover and nitrogen balance: Rates of whole body protein turnover, synthesis and degradation were calculated from the plateau isotopic enrichment of urinary urea following a primed, constant 24-hr infusion of ^{15}N-glycine as described in Chapter 2. Daily nitrogen balance was estimated by subtracting total urinary nitrogen plus 2gN (as an estimate of stool and skin nitrogen losses) from daily nitrogen intake.

Statistical Analysis: Results are reported as means ± standard error of the mean. Tests yielding results suitable for paired comparison were assessed by Student's paired t-test.

Results

Effect of feeding on patient weight and performance status: The weight and performance score of individual patients at the start of naso-gastric feeding and after administration of the normal and ketogenic diets are shown in Table 9.3. The mean weight of the patients had not changed significantly after six days of normal diet (38.2 ± 3.4 cf 38.6 ± 3.8kg : $P < 0.1$). However, following seven days of the ketogenic diet the weight of the patients had increased by approximately 2kg ($P<0.05$). Similarly, the performance score (W.H.O. grading system) of the patients did not change during the period of the normal diet but had improved by one grade in all individuals by the end of the study.

Effect of ketogenic diet on various substrate concentrations in blood: The mean concentration of various substrates in the blood of patients whilst on the normal diet (day 5) and at several times whilst on the ketogenic diet are shown in Table 9.4. A significant ketosis (acetoacetate plus 3-hydroxybutyrate > 0.2 mmol/l) was observed in patients fed for only 24 hours on the ketogenic diet (total blood ketones concentration on day 8: 0.83 mmol/l) and this level increased to a total blood ketone concentration of 1.21 mmol/l after seven days. In contrast, there was a 14% decrease in the blood glucose concentration by the seventh day of the ketogenic diet compared with the concentration observed when the patients were fed on the normal diet (5.7 ± 0.5 cf 6.6 ± 0.5 mmol/l : $P < 0.05$). Similarly, by the seventh day of the ketogenic diet the mean concentration of blood lactate had

Patient	Start of Study Weight (kg)	PS*	End of Normal Diet Weight (kg)	PS*	End of Ketogenic Diet Weight (kg)	PS*
1	32.0	3	32.5	3	34.5	2
2	45.0	3	45.0	3	47.0	2
3	35.0	4	33.5	4	38.0	3
4	31.0	3	32.2	3	33.0	2
5	50.0	4	48.0	4	50.0	3
mean	38.6		38.2		40.5	
(sem)	(3.8) a_1		(3.4) a_2		(3.4) b	

PS* = performance score (WHO system)
b v a_1 or a_2 : $P < 0.05$.

TABLE 9.3 Weight and performance score of patients before start of nasogastric feeding, at the end of six days of normal diet and after 7 days of ketogenic diet.

Substrate (mmol/l)	Normal Diet	Ketogenic Diet		
	Day 5*	Day 8*	Day 10*	Day 13*
Acetoacetate	0.04 (0.01)a	0.35 (0.07)	0.37 (0.14)	0.32 (0.07)b
3-Hydroxybutyrate	0.04 (0.01)a	0.48 (0.11)	0.47 (0.21)	0.89 (0.33)b
Total ketones	0.07 (0.01)a	0.82 (0.16)	0.83 (0.27)	1.21 (0.33)b
Glucose	6.6 (0.5)a	6.5 (0.6)	5.9 (0.6)	5.7 (0.1)b
Lactate	1.3 (0.2)c	0.7 (0.1)	0.6 (0.1)	0.7 (0.1)d
Pyruvate	0.10 (0.01)a	0.08 (0.01)	0.08 (0.01)	0.06 (0.01)b
Free Fatty Acids	0.21 (0.07)	-	-	0.40 (0.11)
Alanine	0.29 (0.05)	-	-	0.18 (0.02)
Glutamine	0.45 (0.05)	-	-	0.41 (0.03)

*time from start of study (days)
b v a : P<0.05
d v c : P<0.01

TABLE 9.4 Effect of ketogenic diet on the concentration in blood of various substrates. Results are mean (SEM), n = 5.

fallen by 46% (0.7 ± 0.1 cf 1.3 ± 0.2mmol/l : P < 0.01) and that of pyruvate by 40% (0.06 ± 0.01 cf 0.10 ± 0.01mmol/l : P < 0.05). Although the mean concentration of blood alanine was decreased by 38% (0.18 ± 0.02 cf 0.29 ± 0.05mmol/l) this difference was not statistically significant. There was no change in the blood glutamine concentration (0.45 ± 0.03 cf 0.41 ± 0.03mmol/l). The mean free fatty acid concentration increased from 0.21mmol/l to 0.40mmol/l but again this difference not statistically significant.

Effect of ketogenic diet on blood insulin concentration: The mean concentration of insulin in blood taken when the patients were on the normal diet (day 5) was not significantly different for that when they were on the ketogenic regimen (day 13) (14.3 ± 1.5 cf 15.3 ± 5.1 uU/ml : P > 0.1)

Effect of systemic ketosis on urea, electrolytes and liver function tests: The mean concentration of urea and electrolytes before feeding was started, and at the end of both the normal diet of the ketogenic diet are shown in Table 9.5. During the period of feeding with the normal diet the mean serum sodium concentration decreased (135 ± 1 cf 130 ± 2mmol/l : P < 0.05) to below the normal range (N.R. 135 - 144mmol/l) but increased again during the period of feeding with the ketogenic diet (130 ± 2 cf 137 ± 2mmol/l : P < 0.05). In contrast, the mean serum potassium concentration was within the normal range throughout the study. There was no significant change in the serum chloride concentration.

The mean concentration of serum bicarbonate remained within the normal range (N.R. 22 - 32mmol/l) throughout the study. However, the concentration decreased significantly during the period of feeding

	Na	K	Cl	HCO_3	Urea	Creatinine (μmol/l)	Ca	PO_4
Normal Range (mmol/l)	(135-144)	(3.5-5.1)	(97-108)	(22-32)	(2.5-7.5)	(60-110)	(2.2-2.6)	(0.8 1.5)
Prior to study	135[a] (1)	3.9[a] (0.2)	98[a] (3)	27 (4)	5.4 (1.2)	77 (6)	2.3 (0.1)	1.1[a] (0.1)
Normal diet	130[b] (2)	4.6[b] (0.3)	103[b] (2)	22 (2)	7.5 (0.6)	59 (9)	2.1 (0.1)	0.5[b] (0.1)
Ketogenic diet	137[c] (2)	3.7[c] (0.3)	103[c] (2)	27 (1)	6.4 (0.3)	57 (4)	2.2 (0.1)	1.0[c] (0.1)

a v b : $P < 0.05$, c v b : $P < 0.05$

TABLE 9.5 Urea and electolytes of patients before nasogastric feeding and at the end of feeding both with the normal diet (day 6) and with the ketogenic diet (day 13). Results are mean (SEM), n = 5.

with the normal diet (27 ± 4 cf 22 ± 2mmol/l : $P < 0.05$), but returned to the initial concentration by the end of the ketogenic diet. There was no significant alteration in the serum concentration of urea and creatinine. The serum phosphate concentrations fell by 50% during the period of feeding with the normal diet (1.1 ± 0.1 cf 0.5 ± 0.1mmol/l : $P < 0.05$) but returned to the initial concentration by the end of feeding with the ketogenic diet.

The liver function tests of patients before the start of nasogastric feeding and at the end of feeding with the normal and ketogenic diets are shown in Table 9.6. The mean concentrations of serum bilirubin, asparate aminostransferase (AST) and alanine aminotransferase (ALT) remained within the normal range throughout the study. Before the start of nasogastric feeding the mean serum albumin concentration was below the normal range (31 - 50g/l) and further decreased during the period of normal diet (31 ± 2 cf 26 ± 2g/l : $P < 0.05$). However, the concentration increased to 29 ± 5g/l by the end of feeding with the ketogenic diet.

Effect of systemic ketosis on daily nitrogen balance and whole body protein turnover: Daily total urinary nitrogen excretion and calculated daily nitrogen balance throughout the periods of normal and ketogenic diets are shown in Table 9.7. The mean daily urinary nitrogen excretion when the patients were fed on the normal diet was 22% less than when the patients were fed on the ketogenic diet (9.5 ± 0.7 cf 11.6 ± 0.5gN.d : $P > 0.1$). Consequently, the mean daily nitrogen balance was slightly but not significantly more positive when the patients were fed on the normal diet as opposed to the ketogenic diet (+6.4 ± 1.8 cf +4.3 ± 1.2gN/d : $P > 0.1$).

Time (Normal Range)	Bilirubin (umol/l) (3-18)	AST (u/l) (10-35)	ALT (u/l) (10-50)	Albumin (g/l) 36-50)
PRIOR TO STUDY	14 (5)	16 (2)	15 (2)	31 (2)a
NORMAL DIET	6 (1)	24 (4)	30 (8)	26 (2)b
KETOGENIC DIET	5 (1)	17 (5)	15 (5)	29 (5)

b v a : $P < 0.05$

TABLE 9.6 Liver function tests of patients before nasogastric feeding and at the end of feeding with the normal diet (day 6) and the ketogenic diet (day 13). Results are mean (SEM), n = 5.

NORMAL DIET			KETOGENIC DIET		
Time* (days)	24hr Total urinary N	Daily Nitrogen Balance (gN/d)	Time* (days)	24hr Total urinary N	Daily Nitrogen Balance (gN/d)
1	8.5 (1.8)	+7.5 (1.6)	7	9.5 (2.4)	+6.4 (1.8)
2	7.4 (1.0)	+8.5 (1.1)	8	12.2 (3.6)	+3.8 (2.1)
3	8.2 (2.1)	+7.7 (1.4)	9	11.9 (3.8)	+4.1 (1.8)
4	11.4 (3.3)	+4.5 (1.3)	10	12.3 (3.8)	+3.7 (1.8)
5	11.7 (2.7)	+4.2 (2.4)	11	13.2 (1.7)	+2.7 (1.2)
6	9.8 (1.4)	+6.1 (3.3)	12	11.0 (2.5)	+5.0 (1.8)
			13	11.2 (2.1)	+4.7 (1.1)
mean (SEM)	9.5 (0.7)	6.4 (1.8)		11.6 (0.5)	4.3 (1.2)

*time from start of study

TABLE 9.7 Daily urinary nitrogen excretion and nitrogen balance of patients during intake of normal (days 1 - 6) and ketogenic diets (days 7 - 13). Results are mean (SEM), n = 5.

Individual and mean rates of whole body protein turnover, synthesis and degradation on the last day of the normal diet and on the last day of the ketogenic diet are shown in Table 9.8. Although the rates of protein turnover, synthesis and degradation of some individuals changed by as much as 60% between the normal diet and the ketogenic diet, there was no overall trend up or down. Moreover, the mean rates of whole body protein turnover, synthesis and degradation when the patients were on either the normal or the ketogenic diets were not significantly different.

Discussion

These results demonstrate clearly that a ketogenic diet containing 70% medium chain triglyceride and supplemented with 4mmol/kg/d 3-D(-)-hydroxybutyrate (arginine salt) can induce a ketotic state in cachectic cancer patients and can maintain these individuals in a positive nitrogen balance.

Prior to the start of nasogastric feeding the patients had lost approximately 32% of their pre-illness stable weight and had been losing weight at the mean rate of 1.8Kg/month (Table 9.1). The patient's weight did not change significantly during the six days when they were given the normal diet but did increase by about 2Kg following the Ketogenic diet.(Table 9.3). The alterations in body composition which might account for this weight gain are not known. However, throughout the study the patients were in a positive nitrogen balance (Table 9.7). Since there was no significant alteration in the blood urea concentration (Table 9.5) this would suggest that at least a component of the weight gain was due to an increase in body protein mass. However, it cannot be established from this study to what extent the weight gain was specifically associated with ketogenic diet.

	NORMAL DIET			KETOGENIC DIET		
Patient	Turnover (gP/Kg/d)	Synthesis (gP/Kg/d)	Degradation (gP/Kg/d)	Turnover (gP/Kg/d)	Synthesis (gP/Kg/d)	Degradation (gP/Kg/d)
1	14.1	12.7	11.2	11.6	9.7	8.9
2	5.7	4.7	2.8	9.1	7.5	6.3
3	10.1	8.0	7.2	6.3	4.8	3.6
4	9.6	8.0	6.7	10.3	8.5	7.6
5	7.0	5.1	4.1	7.5	5.0	4.6
mean	9.3	7.7	6.4	9.0	7.1	6.2
(SEM)	(1.4)	(1.4)	(1.5)	(0.9)	(0.9)	(0.9)

Table 9.8 Whole body protein turnover, synthesis and degradation of patients on final day of normal diet and of ketogenic diet (n = 5)

The reduction in serum sodium and albumin concentrations (Tables 9.5 and 9.6 respectively) which occurred during the period of feeding with the normal diet were reversed during the period of feeding with the ketogenic diet. These effects could be explained by an initial gain and subsequent loss of water from the extracellular fluid space. It is noteworthy that the gain in body weight coincided with the rise in serum sodium and albumin concentrations suggesting that the dilutional effect observed in the initial phase of refeeding was due to a fluid shift from the intracellular space into the extracellular space rather than a net fluid retention. This was probably caused by the initiation of refeeding in very wasted patients rather than any effect specifically related to the normal or ketogenic diets. The fall in serum phosphate concentration during the normal diet probably reflects an initial uptake of phosphate into cells associated with the anabolic stimulus of refeeding.

Blood glucose lactate, and pyruvate concentrations decreased and there was a trend towards a reduced plasma alanine with the establishment of systemic ketosis (Table 9.4). The rate of hepatic gluconeogensis is, to some extent, controlled by the concentration of available substrates (Newsholme, 1976). Since lactate, pyruvate and alanine can act as substrates for glucneogenesis it is possible that the changes in substrate concentrations associated with systemic ketosis might reduce the rate at which glucose is supplied to the tumour. Moreover, the trend towards elevated blood levels of fat-derived energy substrates (free fatty acids and ketone bodies) and reduction in blood glucose concentration (Table 9.4) emphasise the potential of systemic ketosis in maintaining the supply of energy to the host whilst reducing glucose supply to the tumour. However, as discussed previously, a dietary induced ketosis did not reduce the blood glucose concentration in rats bearing the Walker 256 carcino-

sarcoma (see Chapter 8). Thus the effects of systemic ketosis on blood substrate concentrations may depend on the individual characteristics of the tumour and host.

The mean nitrogen balance of the patients was positive throughout the study and was not significantly different when the patients were on the ketogenic diet compared with the normal diet. The observation that the patients were in a positive nitrogen balance whilst on both diets probably reflects that prior to the initiation of nasogastric feeding their food intake was markedly reduced. Thus, added dietary energy and protein were taken up by body tissues irrespective of the composition of the diet. The positive nitrogen balance of the cancer patients whilst on the ketogenic diet does, however, contrast with the findings of a recent study which examined the metabolic effects of dietary induced ketosis in young male volunteers (Phinney et al., 1983). The diet in the latter study provided 85% of calories as fat, 1.75g of protein per kilogram per day and less than 20g of carbohydrate per day. With this regimen the volunteers demonstrated a negative nitrogen balance during the first seven days of the ketogenic diet but achieved nitrogen balance thereafter. This initial negative balance was probably due to this use of amino acids from muscle protein as substrates for hepatic gluconeogenesis. Such de novo synthesis of glucose is required to support glucose dependent tissues such as the brain and red blood cells. It is only with the production of ketone bodies from fat and their use as energy substrates instead of glucose that this nitrogen efflux is reduced, as in compensated starvation (Robinson and Williamson, 1980). However, in this study, the patients remained in a positive nitrogen balance throughout the induction of ketosis (Table 9.7). One explanation could be that the diet provided 30 - 40g of carbohydrate per day and consequently the requirement for extra glucose

production via gluconeogenesis was minimal. Alternatively, the infused 3-hydroxybutyrate may have been utilized as an alternative substrate to glucose in the interval between the time of glucose deprivation and the onset of adequate hepatic ketogenesis.

The mean rates of whole body protein turnover, synthesis, and degradation were similar when patients received either the normal or the ketogenic diets (Table 9.8). Since the nitrogen balance of the patients was similar during either regimen (Table 9.7), these findings suggest that in cancer cachexia, a dietary induced systemic ketosis does not influence either whole body nitrogen kinetics or the ability of the body to retain nitrogen. Whether the observed changes in energy substrate concentrations might lead to a reduction in tumour growth rate and thereby influence the development of cachexia in the long term remains to be determined.

Summary and Conclusions

The aim of this study was to determine whether a ketogenic diet might improve host nitrogen balance whilst reducing the supply of glucose for tumour energy metabolism in humans. Five patients with malignant disease and severe weight loss (mean 37%) were fed via a fine bore nasogastric tube. A normal diet was given for six days and this was followed by seven days of an isonitrogenous, isocaloric, ketogenic diet.

Both diets were well tolerated. At seven days the mean ketone body concentration in the blood of patients fed the ketogenic diet was 1.21 $\pm$ 0.33mM. This ketosis was associated with a significant reduction of the concentration in blood glucose, lactate and pyruvate.

There was, however, no significant alteration in host nitrogen balance or whole body protein synthesis, degradation or turnover rates when patients changed from the normal to the ketogenic diet.

Thus, although the ketogenic diet was well tolerated there was no beneficial effect on the nitrogen metabolism of the cachectic cancer patients. Whether the change from glucose to fat derived energy substrates might reduce tumour growth rates in the long term remains to be determined.

Selective inhibition of tumour energy metabolism in vivo

Introduction

From Chapter 8 it would appear that control of tumour growth is a prerequisite for effective nutritional therapy in cancer cachexia and this has been suggested previously (Van Eys, 1982). Current cancer chemotherapy involves the use of cytotoxic agents to inhibit cell division within the tumour. These agents are non- selective since they also inhibit cell division within the tissues of the host. Their use is based upon the assumption that the rate of cell division within the tumour is greater than that of the majority of host tissues. Clearly this is not always a valid assumption (Calman, Smyth and Tattersall, 1980) and such treatment is limited by host toxicity, some of which is manifest by a deterioration in the nutritional status of the patient (Kokal, 1985). In the cachectic cancer patient antineoplastic therapy has to be effective yet non-toxic. A more selective and thus less toxic approach to the treatment of cancer might be based on differences between tumour and host cells. One such difference is the altered energy metabolism of many tumour cells.

Mitochondria of normal cells: Every eukaryotic cell contains mitochondria. The Greek words from which mitochondria get their name (mitos-thread; chondros-granule) characterise the appearance of these organelles under the light microscope. However, under the electron microscope mitochondria have a highly organised structure. Two membranes surround the inner matrix. The smooth outer membrane is porous to molecules up to a molecular weight of 8,000 (Zalman, Nikaido and Kagawa, 1980). The inner membrane is plicated to form cristae and

acts as a semipermeable membrane. The primary function of mitochondria is to couple the degradation of various organic compounds to the formation of ATP. Hence, they have been regarded as the 'power houses' of the cell. Mitochondria are also remarkable because they are in possession of their own genetic system (Kroon and Saccone, 1980) and replicate in a manner similar to that of the binary fission of bacteria. However, although mitochondria are able to synthesise some of their own constituent proteins (mainly those bound to the inner membrane) the nuclear genetic system is responsible for the majority of the proteins within these organelles.

Mitochondria contain numerous enzymes which provide the cell with reducing equivalents and metabolic intermediates. They contain a system for the oxidative degradation of fatty acids, the B oxidation pathway (Greville and Tubbs, 1968), and also possess enzymes for the oxidative decarboxylation of some amino acids. In addition, mitochondria contain the Krebs cycle enzymes (Lowestein, 1969) which degrade acetyl-CoA. Acetyl CoA is formed as a result of the oxidative degradation of lipids, some amino acids and carbohydrates. The processing of these substrates along such catabolic pathways leads to the production of reducing equivalents in the form of NADH and reduced flavins. The availability of reducing equivalents is required for the formation of ATP during oxidative phosphorylation.

The respiratory chain is a multienzyme system which is firmly embedded in the inner mitochondrial membrane. The chain is composed of flavoproteins, iron-sulphur proteins, ubiquinone and several cytochromes. Reducing equivalents, formed during various oxidation reactions occurring inside as well as outside the mitochondria, are transferred along this chain to molecular oxygen. The hydrogen atoms,

or the electrons, are thus passed from the negative redox potential of their initial acceptors to the positive potential of oxygen. This fall of free energy of electrons is the primary force for ATP synthesis (oxidative phosphorylation).

Mitochondria of tumour cells: Almost 60 years ago Warburg hypothesised that damage to respiration, that is a deficiency in the respiratory chain, causes the transformation of normal cells into tumour cells (Warburg, 1930). However, tumour mitochondria generally contain normal, funtional respiratory chains. It is possible to quantify the efficiency of ATP synthesis by mitochondria. If a known amount of ADP is added to isolated mitochondria, the amount of oxygen consumed in order to convert the ADP to ATP can be determined and is known as the P/O ratio. Carefully isolated tumour mitochondria show normal P/O ratios and normal respiratory control indices (Pederson, 1978). A general disorder of tumour mitochondria is therefore unlikely to be the major underlying factor in the neoplastic transformation process (Weinhouse, 1982). Nonetheless, an extensive literature has accumulated to show that tumour cell mitochondria have aberrations of one kind or another. These include deficiencies of enzymes involved in the metabolism of ketone bodies (Tisdale and Brennan, 1983; see also Chapter 8), alterations in inner membrane components particularly phospholipids (Reitz, Thomson and Morris, 1977) abnormal adenine nucleotide translocation (Lau and Chan, 1984), and alteration of respiratory chain components (Nelson et al., 1984).

Whatever the significance of these mitochondrial alterations in terms of carcinogenesis, it is clear that in a great many cancers the organelle is defective. From the viewpoint of cancer chemotherapy, this would seem to be a weak spot in cancer cells and could be the

focal point of therapy using antimitochondrial agents. Indeed various anticancer drugs have been shown to inhibit mitochondrial function (Gonsalvez et al., 1976; Bernal, Shapiro and Chen, 1982) and several inhibitors of mitochondrial function have been demonstrated to have selective antineoplastic effects (Wilkie, 1979). However, as yet the use of such agents to inhibit selectively mitochondrial function and thereby reduce tumour growth rate has received little or no attention in clinical cancer chemotherapy.

High glycolytic capacity of tumour cells: An increased rate of anaerobic and aerobic glycolysis has been demonstrated repeatedly in rapidly growing tumours (Warburg, 1930; Weber et al., 1961). Moreover many tumours have been shown to have a high rate of glucose consumption and to be susceptible to carbohydrate deprivation (Demetrakopoulos, Linn and Amos, 1978). The increased glycolysis of some tumours has been linked to enhanced levels of glycolytic-enzymes (Weber and Morris, 1963). These appear largely as fetal-like isoenzymes (Ibsen, 1977; Weber, 1977) which are less prone to product inhibition (Bustamante and Pederson, 1977).

The increased glycolysis of tumour cells has prompted several attempts to reduce carbohydrate availability or utilization in the hope that such intervention might preferentially impair malignant cells. The glucose antimetabolite 2-deoxyglucose has been shown to reduce the growth rate of various rodent tumours in vivo (Ball, Wick and Sanders, 1957; Sokoloff et al., 1955) and in tumour bearing rats inhibition of gluconeogenesis with hydrazine sulphate has been shown to have antineoplastic activity (Gold, 1971). Furthermore, hydrazine sulphate can enhance the efficacy of conventional cytotoxic therapy (Gold, 1975). However, although both 2-deoxyglucose (Landau et al., 1958) and

hydrazine sulphate (Lerner and Regelson, 1976) have been administered to cancer patients, clinical efficacy has not been demonstrated conclusively.

A series of experiments involving the mitochondrial dye Rhodamine 1,2,3 has recently given new impetus to the use of tumour energy metabolism as a target for systemic therapy in cancer. Rhodamine 1,2,3 and Rhodamine 6G are two related compounds which have been shown to be cytotoxic in vitro (Zigman and Gilman, 1980; Johnson, Summerhayes and Chen, 1982; Wilie and Fearon, 1985). They belong to a series of permeant cationic fluorochome dyes with a high negative reduction potential which inhibit both protein and DNA synthesis in intact cells and also delay mitosis (Zigman and Coilman, 1980). Both agents are potent inhibitors of mitochondrial oxidative phosphorylation (Higuti et al., 1980; Lampidis et al., 1983) and it has been suggested that their cytotoxic activity is related to an inhibition of ATP production (Darzynkiewicz et al., 1982).

Rhodamine 1,2,3 is taken up into the mitochondria of both normal and tumour cells but is retained for much longer in those of the tumour cells. This retention is associated with the cytotxic action of the dye. In addition, Rhodamine 1,2,3 has been shown to be selectively cytotoxic against numerous malignant cell lines (Lampidis et al., 1983). This selectivity is thought to be related to a transformation dependent abnormality of mitochondrial membrane potential (Nadakavukaren, Nadakavukaren and Chan, 1985). If the glucose antimetabolite 2-deoxyglucose, is administered simultaneously, the two agents have been shown to have synergistic anti-tumour activities both in vitro (Lampidis et al., 1983) and in vivo (Bernel et al., 1983). Thus it would appear that much greater efficacy can be achieved if both glycolysis and oxidative phosphorylation, the two main intracellular sources of ATP, are inhibited simultaneously.

An alternative method of reducing glycolysis would be to induce a state of hypoglycaemia. This can be achieved by inhibiting gluconeogenesis in the fasting state. The present investigation was undertaken to examine the effects of an antimitochondrial agent, an inhibitor of gluconeogenesis, and the combination of the two on the growth rate of the Walker 256 carcinosarcoma in rats. Rhodamine 6G is a fluorochrome mitochondrial dye (Higuti et al., 1980) and has been shown to be a more potent inhibitor of tumour cell growth in vitro than Rhodamine 1, 2, 3 (Wilkie and Fearon, 1985). Hypoglycaemia was induced with 3-mercaptopicolinic acid. This compound blocks the synthesis of glucose de novo by inhibiting phosphoenolpyruvate carboxykinase (E.C.4.1.1.32) (Di Tullio et al., 1974) and has been shown to induce marked hyopglycaemia in both fasted rats (Di Tullio et al., 1974) and man (Burt et al., 1985).

Materials and Methods

Chemicals: 3-Mercaptopicolinic acid was a gift from Dr N.W. Di Tullio, Smith, Klyne and French Ltd, Philadelphia, USA. Rhodamine 6G was obtained from BDH Chemicals Ltd, Poole, Dorset, England.

Animals: Inbred female Wistar rats, aged between 12 -16 weeks and weighing 180 - 220g were allowed free access to food (SDS diet, SDS Ltd., Witham, Essex) and water except where stated. Animals were kept in conditions of controlled temperature and lighting ($20^{o}C \pm 2^{o}C$, 12h/12h light/dark cycle).

Tumour: The rapidly growing Walker 256 carcinosarcoma described in Chapter 7 was used. Viable tumour fragments (100mg) were transplanted subcutaneously into the right flank of rats under aseptic conditions

and light ether anaesthesia. The tumour doubling time was about 36h.

Cannulation of rats for serial measurement of blood glucose concentration: The right common carotid artery was cannulated 24 hours prior to drug administration under aseptic conditions and halothane/nitrous oxide anaesthesia. The cannula (1mm external diameter Portex polythene tubing, Portex Ltd, Hythe, Kent, England) was heparinised (5000u/ml) and then externalised onto the dorsal aspect of the animals neck via a subcutaneous tunnel. Once outside the animal, the cannula was protected by a tightly coiled stainless steel spring. The spring and cannula were suspended from a pulley system which allowed the animal to move freely within a wire bottomed, perspex metabolism cage.

Serial blood samples (100ul) were obtained via the cannula and the total blood volume of the rats was maintained by replacing each blood sample with an equal volume of isotonic saline (0.9% W/V Na Cl).

Measurement of blood glucose concentration: The concentration of glucose in blood was determined by an enzymatic method (GOD - Perid, Boehringer Mannheim, Lewes, Sussex, England). 100ul blood samples were immediately deproteinised with 1ml of uranyl acetate solution (Boehringer Mannheim) and then centrifuged. The supernatant was stored at $4^{o}C$ and assayed on the same day.

Drug Administration Animals underwent tumour implantation 48h prior to i.p. drug administration. The hypoglycaemic effect of 3MPA was only observed in the fasted state, therefore except where stated rats were fasted for 24h prior to drug administration. The animals were then fasted for a further 8h before being allowed access to food.

Statistical analysis Overall statistical significance was determined by an analysis of variance. Dunnett's test (1955) was used when multiple comparisons were made within each group.

Results

Effect of 3MPA and R6G on blood glucose concentration of starved normal rats: The blood glucose concentration of control rats fasted for 24 hours was approximately 3.5mmol/l and remained constant throughout the seven hour sampling period (Figure 10.1). Administration of 3MPA (70mg/kg) to rats fasted for 24h resulted in a signficant decrease in the blood glucose concentration ($P < 0.01$) within 15 minutes. This decrease continued and reached a minimum level of 1.4mmol/l after 180 minutes. Thereafter, there was a gradual increase in blood glucose concentration until normal fasting values were achieved 360 minutes after drug administration (Figure 10.1).

Rats given both 3MPA (70mg/kg) and R6G (0.8mg/kg) had significantly lower mean blood glucose concentrations at 120 and 180 minutes compared with rats given 3MPA (70mg/kg) alone (Figure 10.2 cf Figure 10.1, $P < 0.01$). However, this decrease was irreversible. The combination of a lower dose of 3MPA (40mg/kg) and the same dose of R6G (0.8mg/kg) induced a reversible period of hypoglycaemia lasting approximately 240 minutes with a trough blood glucose concentration of 2.2mmol/l at 180 minutes (Figure 10.2).

Effect of 3MPA and R6G on blood glucose concentration of fed and starved tumour bearing rats: The mean blood glucose concentration of 24h fasted tumour bearing rats was 2.3 ± 0.3mmol/l (Figure 10.3) 180 minutes after injection of 3MPA (40mg/kg) plus R6G (0.8mg/kg). In

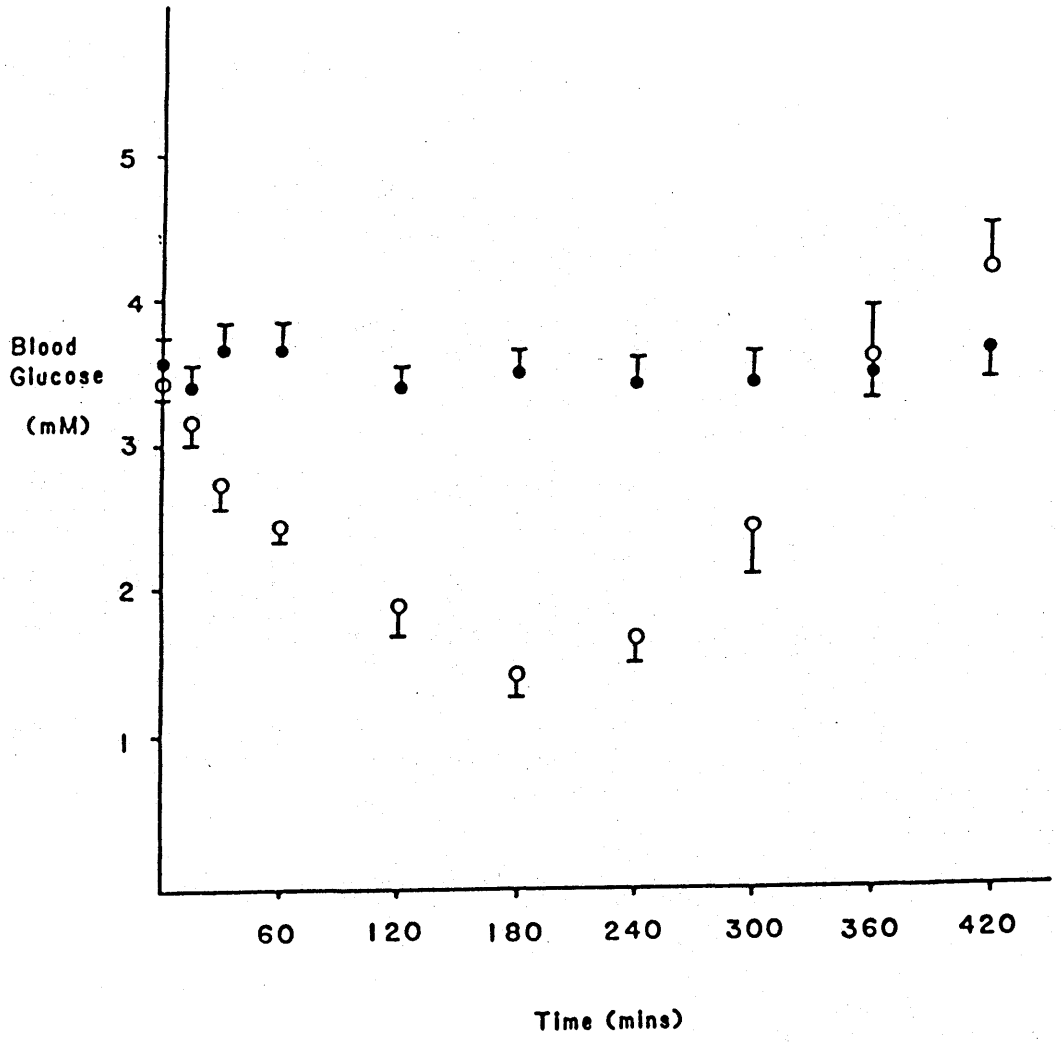


Figure 10.1 Effect of 3MPA on blood glucose concentration of starved non-tumour-bearing rats. Rats (8 per group) were starved for 24h prior to, and for 8h after administration i.p. of saline (solid circles) or 3MPA at a dose of 70mg/Kg (open circles). Repeated blood samples were obtained from an indwelling arterial cannula at the times indicated. Values are mean ± SEM.

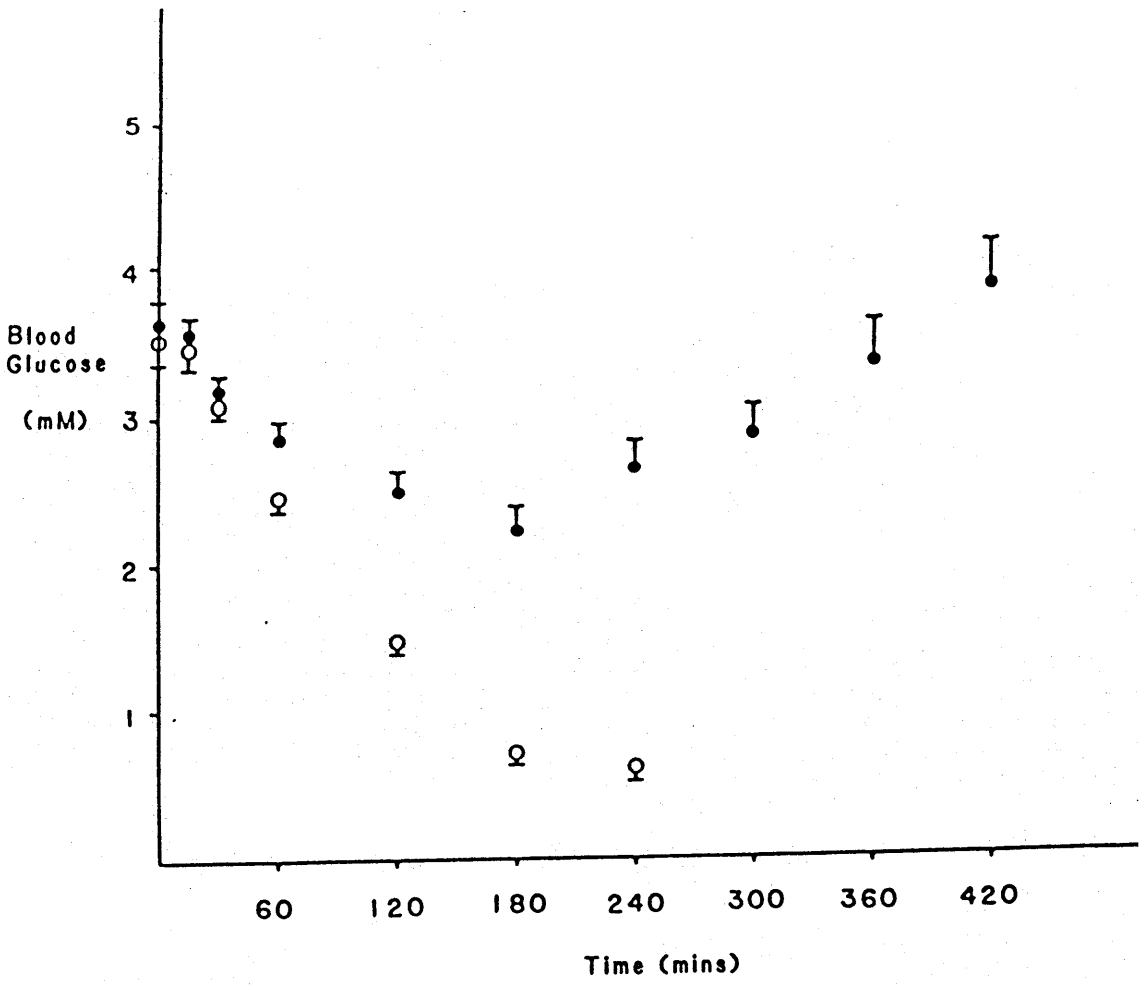


Figure 10.2 Effect of combination of 3MPA plus R6G on blood glucose concentration of starved non-tumour-bearing rats. Rats (8 per group) were starved for 24h prior to, and for 8h after administration i.p. of 3MPA (40mg/Kg) (solid circles) or 3MPA (70mg/Kg) plus R6G (0.8mg/Kg) (open circles). Repeated blood samples were obtained from an indwelling arterial cannula at the times indicated. Values are mean ± SEM.

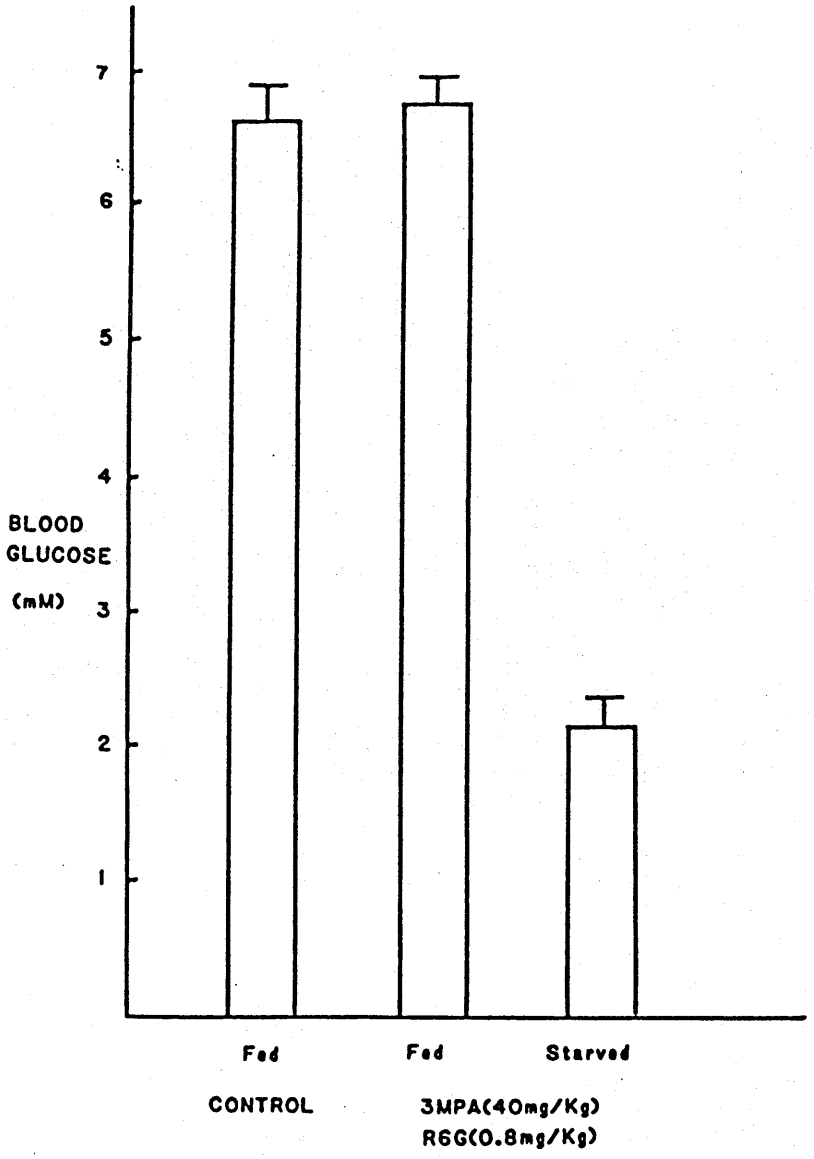


Figure 10.3 Effect of 3MPA plus R6G on blood glucose concentration of non-tumour-bearing rats fed ad libitum or starved for 24h. 180 minutes after drug administration i.p. rats were lightly anaesthetised and blood was obtained by cardiac puncture for estimation of blood glucose concentration. Values are mean ± SEM.

contrast, the mean blood glucose concentration of rats fed ad libitum and given 3MPA (40mg/kg) plus R6G (0.8mg/kg) was 6.7 ± 0.3mmol/l (Figure 10.3). This was not significantly different from controls fed ad libitum (6.6 ± 0.3mmol/l, Figure 10.3).

The fasting blood glucose concentration of tumour bearing rats was not significantly different from that of 24h starved non-tumour bearing rats (Figure 10.4). Moreover, the blood glucose concentration in fasted tumour bearing rats 180 minutes after administration of 3MPA (70mg/kg) or 3MPA (40mg/kg) plus R6G (0.8mg/kg) was not significantly different from that of non-tumour bearing rats given the same drug regimen (Figure 10.4).

Effect of R6G on growth rate of Walker 256 tumour in rats: The effect of R6G administration 48h after tumour implantation on tumour weight 5 days after drug administration are shown in Figure 10.5. Host weight loss and mortality in each group is also shown. Administration of R6G (0.8mg/kg) led to a small but not significant reduction of tumour weight (5.6 ± 0.5 cf 7.1 ± 0.7, $P > 0.05$). This dose was associated with a weight loss of 5% compared with 1.5% in the controls. When the dose of R6G was increased to 1.6mg/kg tumour weight was reduced by 30% but again this decrease was not significant (5.0 ± 0.5 cf 7.1 ± 0.7, $P > 0.05$). Host weight loss increased to 9.2% but there was no mortality. At a dose of 2.4mg/kg tumour weight was reduced by 75% (1.8 ± 0.5 cf 7.1 ± 0.7, $P < 0.01$) but one out of seven animals died. At a dose of 3.0mg/kg 3 out of 7 animals died and at 4.0mg/kg all the animals died within 3 days.

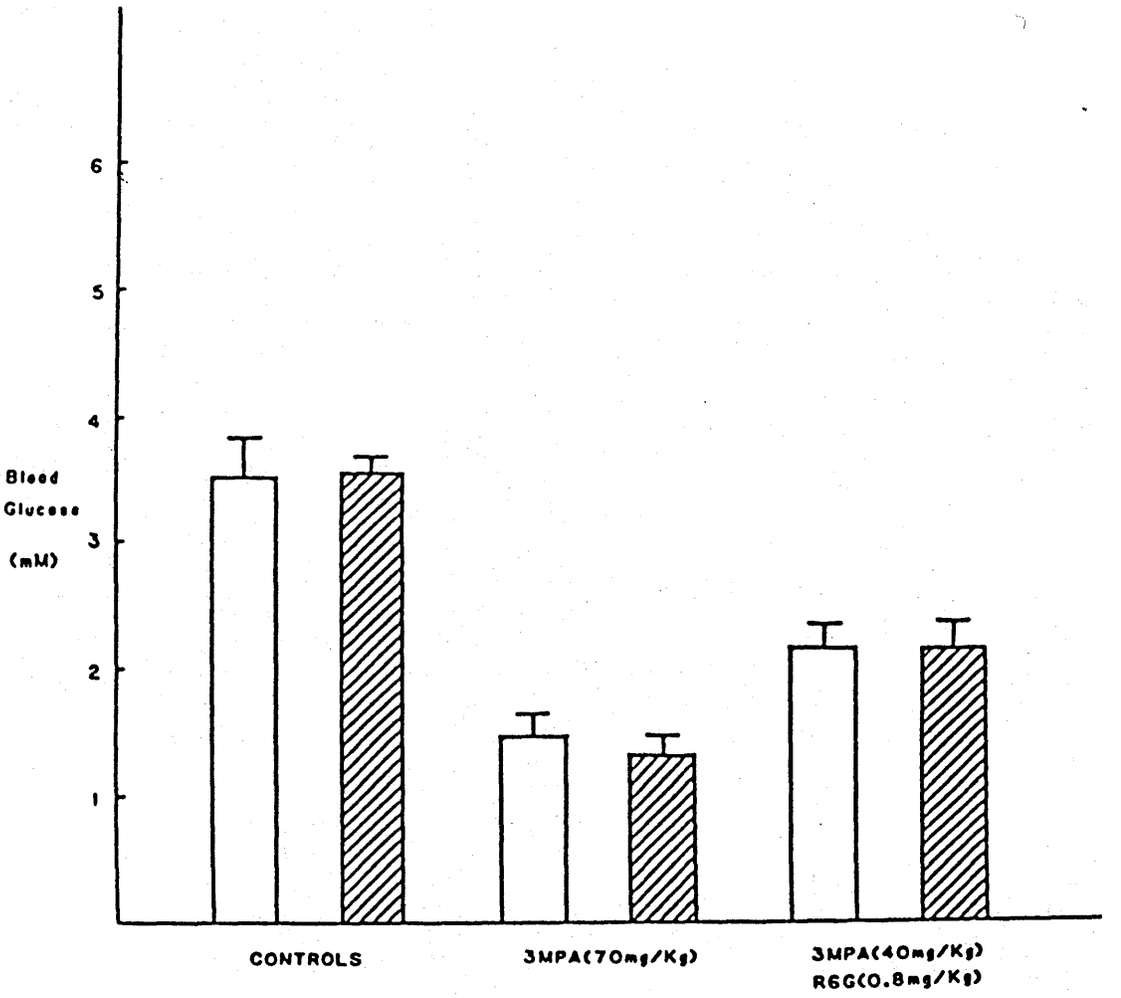


Figure 10.4 Effect of 3MPA alone or 3MPA plus R6G on blood glucose concentration of starved tumour-bearing and non-tumour-bearing rats. Rats (7 per group) were starved for 24h prior to, and for 3h after administration i.p. of either saline, 3MPA (70mg/Kg) or 3MPA (40mg/Kg) plus R6G (0.8mg/Kg). Tumour bearing rats (hatched columns) had undergone implantation of the Walker 256 tumour 48h prior to drug administration. Non-tumour-bearing rats (open columns) had undergone sham operation. 180 minutes after drug administration, all groups of rats were lightly anaesthetised and blood was obtained by cardiac puncture for estimation of blood glucose concentration. Values are mean $\pm$ SEM.

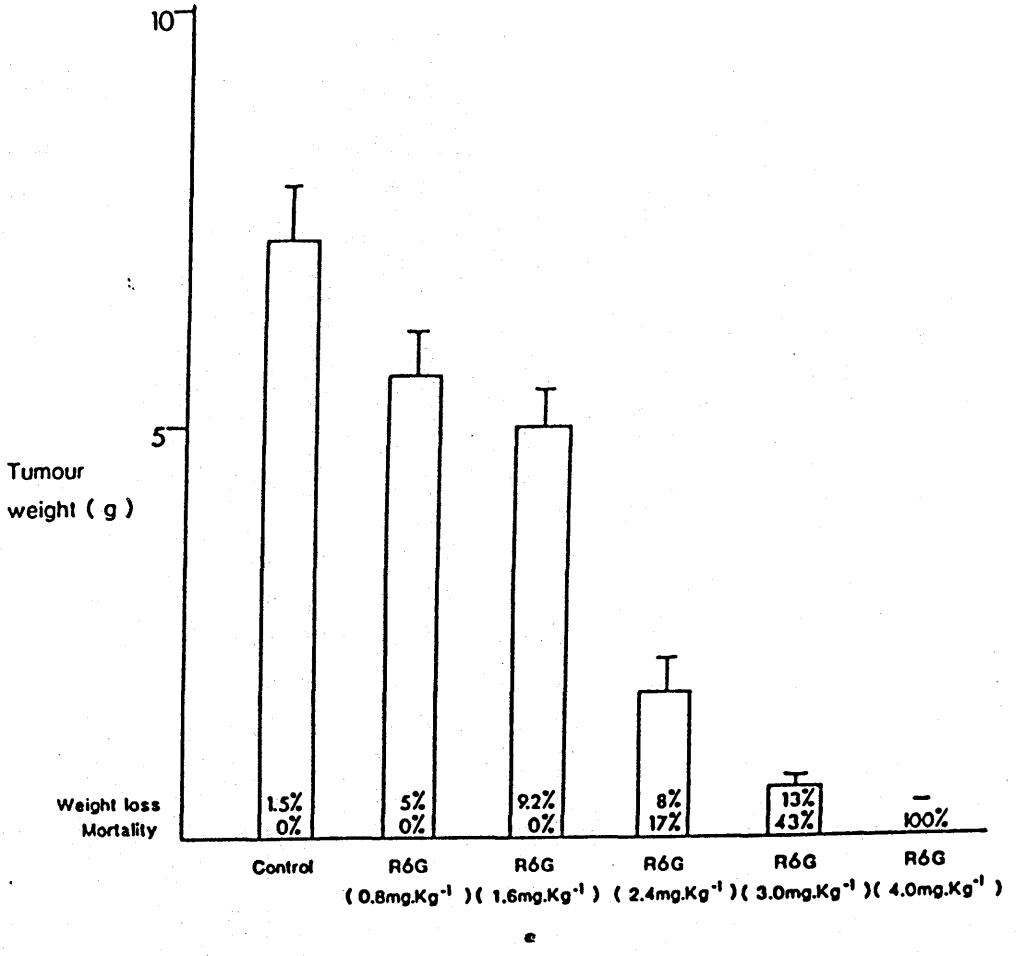


Figure 10.5 Effect of increasing doses of R6G on growth rate of Walker 256 tumour in rats. R6G was administered i.p. at the doses shown 48h after tumour implantation. Five days after drug administration the animals were sacrificed and the tumours were excised and weighed. Values are mean ± SEM. The percentage weight loss of the animals over the seven days of the study is shown within the columns.

Effect of 3MPA plus R6G on growth rate of Walker 256 tumour in fed and fasted rats: When the combination of 3MPA (40mg/kg) and R6G (0.8mg/kg) was administered 48h after tumour implantation, to rats starved for 24h the mean tumour weight 5 days after drug administration was 49% less than that of controls (3.6 ± 0.3 cf 7.1 ± 0.7g, $P < 0.05$). Host weight loss was only 3% in rats receiving combination therapy and was comparable to that in control tumour bearing animals (2%) (Figure 10.6) However, when the combination of 3MPA (40mg/kg) plus R6G (0.8mg/kg) was administered 48h after tumour implantation to rats fed ad libitum the mean tumour weight was not significantly different from that of untreated tumour bearing animals (6.2 ± 0.5 cf 7.3 ± 0.6g, $P > 0.1$, Figure 10.6).

Starvation for a period of 32h commencing 48h after tumour implantation had no effect on tumour weight 7 days after tumour implantation (7.3 ± 0.6 cf 7.1 ± 0.7g, $P > 0.1$). Similarly, administration of 3MPA (70mg/kg) 48h after tumour implantation to rats starved for 24h prior to and for 8h after drug administration had no effect on tumour weight 7 days after implantation (7.4 ± 0.5 cf 7.1 ± 0.7g, $P > 0.1$, Figure 10.6). Administration of R6G (0.8mg/kg) 48h after tumour implantation to rats starved for 24h prior to and 8h after drug administration resulted in a slight but not significant reduction in tumour weight 7 days after implantation (5.6 ± 0.5 cf 71 ± 0.7g, $P > 0.05$, Figure 10.6).

Discussion

This study demonstrates clearly that the growth rate of the Walker 256 in rats is reduced significantly when R6G is administered during a period of hypoglycaemia. Neither hypoglycaemia or R6G alone

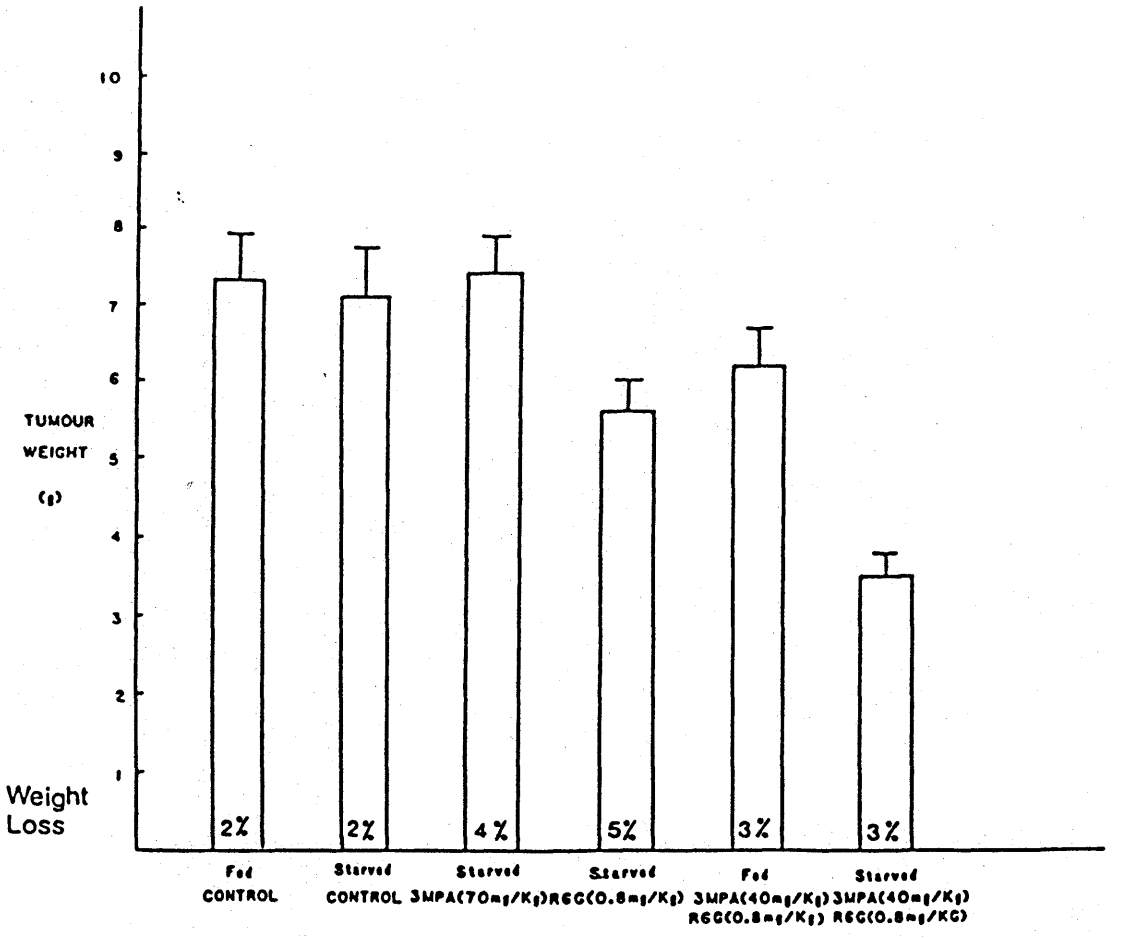


Figure 10.6 Effect of 3MPA plus R6G on growth rate of the Walker 256 tumour in rats fed ad libitum or starved for 24h prior to and for 8h after drug administration i.p. The drug regimens were as shown. Tumours were implanted 48h prior to drug administration and animals were killed 5 days thereafter. Values are mean ± SEM (n=7 per group). The percentage weight loss of the animals over the seven days of the study is shown within the columns.

caused a significant inhibition of tumour growth. The combined regimen was well tolerated and reduced tumour growth rate with minimal host toxicity.

When 2-deoxyglucose (an inhibitor of glycolysis) is administered at the same time as Rhodamine 1, 2, 3 the combination has synergistic antitumour activity both *in vitro* (Lampidus et al., 1983) and *in vivo* (Bernal et al., 1983). This suggests that when combined with selective inhibition of mitochondrial function a reduction in glycolysis may have a potent antitumour effect. In this study increasing doses of Rhodamine 6G were associated with decreased rates of tumour growth. Unfortunately such high doses of R6G were also associated with increasing host weight loss and mortality (Figure 10.5). However, when the circulating concentration of glucose was reduced with 3MPA (Figure 10.6), tumour growth rate was inhibited by a dose of R6G which was not toxic to the host (Figure 10.6).

The hypoglycaemic effect of 3MPA is due to the inhibition of glucose synthesis *de novo* (Di Tullio et al., 1974). Thus hypoglycaemia is only observed in the fasting state because if the animals have free access to food there is usually an adequate supply of glucose from the diet to maintain blood glucose levels. It might be argued that it was not the hypoglycaemic effect of 3MPA which was important in making the R6G/3MPA combination effective. However, the blood glucose concentration of tumour bearing rats starved for 24h and given 3MPA (40mg/kg) plus R6G (0.8mg/kg) was signficantly reduced compared with fasted controls (Figure 10.4) In contrast, there was no reduction of blood glucose concentration (Figure 10.3) or of tumour growth rate (Figure 10.6) in rats given the same combination but fed *ad libitum*.

Thus, a reduction in blood glucose concentration would appear to have been necessary for the combination to be effective.

The effects of starvation on tumour growth rate in vivo appear to vary from one animal model to another. Previous animal studies have demonstrated either no change (Goodgame et al., 1979) or a slight reduction of tumour growth rate (Giovanella et al., 1982) following variable periods and degrees of caloric restriction. In this study, a period of starvation lasting 32h had no effect on final tumour weight (Figure 10.6). Moreover, the administration of 3MPA (40mg/kg) or R6G (0.8mg/kg) to tumour bearing rats starved for 24h before drug administration and for 8h thereafter did not reduce final tumour weight (Figure 10.6). However, when the combination of R6G plus 3MPA was administered to rats starved for a total of 32h final tumour weight was reduced by nearly 50% (Figure 10.6). This supports the conclusion that is was the combination of drugs administered in the fasting state which was important in reducing tumour growth rate.

Several malignant or transformed cell types, when deprived of a glucose source, suffer a dramatic lowering of ATP levels within the first hour of starvation (Demetrakopoulos, Linn and Anlos, 1978). However, normal cells are able to maintain their ATP content for 12 to 24h at levels essentially similar to those of cells grown in the presence of glucose. This differential effect probably arises from the requirement of malignant cells for glucose as an energy source.

Previously it has been demonstrated that the growth rate of the Walker 256 tumour in rats can be reduced by administration of the glucose antimetabolite 2-deoxyglucose (Ball, Wick and Sanders, 1957) or by administration of hydrazine sulphate which inhibits gluconeogenesis (Gold, 1971). This suggests that the Walker 256 tumour is largely

dependent on glucose as an energy substrate. Walker 256 cells have also been shown to possess few mitochondria and those that are present have abnormal morphology and a reduced complement of enzymes (Pedersen, 1978, see also Chapter 8). It is possible that the synergy between R6G and hypoglycaemia was the result of R6G inhibiting an already restricted capacity for ATP production via oxidative phosphorylation. Thus, the tumour would be even more dependent on glycolysis for energy production. In this situation even a small decrease in the substrate available for glycolysis might lead to a substantial reduction in intracellular ATP concentration, thereby reducing cell viability.

Under normal circumstances when 1 mole of glucose is fully oxidized to carbon dioxide and water, 2 moles of ATP are produced via glycolysis and 36 moles are produced from oxidative phosphorylation. Therefore, in terms of ATP production it is much more efficient if glucose is fully oxidized via oxidative phosphorylation rather than partially oxidized to lactate via glycolysis. The AS-30D hepatoma is similar to the Walker 256 tumour in that it is a rapidly growing rodent tumour which is known to exhibit high rates of aerobic glycolysis and lactate production in vitro (Pedersen, 1978). It has been demonstrated that when glucose is supplied to AS-30D cells as the exogenous energy source 60% of total ATP production is derived from glycolysis and only 40% from oxidative phosphorylation (Nakashima, Paggi and Pederson, 1984). However, in the absence of added glucose, glutamine alone can maintain the same ATP production rates. Thus although these hepatoma cells rely predominantly on the inefficient glycolytic mode for energy production in vitro, in the absence of glucose other subtrates which enter the Krebs cycle directly are able to maintain ATP production.

In the present study, a selective antineoplastic effect was demonstrated by administration of an inhibitor of oxidative phosphorylation (R6G) during a period of hypoglycaemia. Neither treatment was effective on its own. This suggests that for the Walker 256 tumour in vivo, both glycolysis and oxidative phosphorylation are important in the maintenance of cellular ATP levels. Furthermore, cancer cell lines exhibit a broad spectrum of aerobic glycolytic activity. Slow growing, well differentiated tumours resemble normal cells in that they exhibit low rates of glycolysis and high rates of oxidative metabolism for energy production (Pedersen, 1978; Weinhouse, 1982). Previous attempts to use tumour energy metabolism as a target for antineoplastic therapy have used single agents aimed at inhibiting either glycolysis or oxidative phosphorylation (Ball, Wick and Sanders, 1957; Wilkie, 1979). Since most tumour cells use both pathways for energy production this approach is unlikely to succeed. The results of this and of other studies (Lamipidis et al., 1983; Bernal et al., 1983) indicate that simultaneous manipulation of both sourses of intracellular ATP may be used to a achieve more selective control of tumour growth.

Summary and Conclusions

The administration of an inhibitor of oxidative phosphorylation (Rhodamine 6G) during a period of hypoglycaemia was shown to reduce significantly the growth rate of the Walker 256 in rats; minimal host toxicity was observed. Neither Rhodamine 6G nor the period of hypoglycaemia alone significantly reduced tumour growth rate. Thus greater antineoplastic activity was achieved by manipulating both oxidative phosphorylation and glycolysis simultaneously. This relatively non-toxic approach may prove useful in the control of tumour growth particularly in the already compromised cachectic cancer patient.

CHAPTER 11

General Discussion and Conclusions

The aims of this thesis have, on the whole, been achieved. The results extend previous studies on the mechanisms of weight loss in cancer cachexia. Studies of the body composition and protein metabolism of cancer patients demonstrate that cachexia is not entirely consistant with semistarvation. However, the apparent lack of an effect of the tumour on host energy expenditure means that the 'metabolic component' of cachexia has yet to be identified. A method of selectively feeding the host but not the tumour was evaluated as a new form of therapy for cachexia. The failure of this approach confirms the conclusion that the best way to cure cachexia is to cure the cancer. Therefore, the most promising finding of this study is a novel form of anticancer therapy. This is based on differences between host and tumour energy metabolism and is shown to be both effective and non-toxic in tumour bearing rats.

The results have been discussed in detail in each Chapter and only the main points will be discussed in this final Chapter.

Clearly an investigation of weight loss must first determine which of the body compartments are reduced. In this study a group of cachectic lung cancer patients who had lost nearly 30% of their usual body weight were shown to have sustained an 80% loss of body fat and a 20% loss of body protein (Chapter 5, Figure 5.1) Since there is no specific store of protein in the body, loss of protein implies loss of structural and functional components of the body. The vital importance of protein is illustrated by the observation that a greater than 30% reduction of body protein is incompatible with survival (Cahill, 1970).

The marked loss of body fat may have been well tolerated but implies that the patients had been in a prolonged negative energy balance. The severe loss of body protein is probably the basic reason for the morbidity and mortality associated with the cachexia of cancer (Warren, 1932; Inagaki, Rodriguez and Bodey, 1974).

Weight loss is most commonly due to a reduced food intake, an increased energy expenditure or a combination of the two. The cachectic patients whose body composition was studied had a markedly reduced food intake (Table 5.4). This would support the observation that in many respects the cachectic cancer patient is suffering from semistarvation (Walsh, Bowman and Jackson, 1983). However, it could not be determined whether a reduced food intake had been present throughout the period of weight loss.

Several studies have examined the dietary intake of weight losing cancer patients at an early stage in the development of cachexia and have failed to demonstrate a significant reduction in food intake (Costa et al., 1981; Burk, Bryson and Kark, 1980; Warnold, Lundholm and Scherston, 1978). It might be argued that failure to detect a difference was simply due to substantial interindividual variation (Acheson et al., 1980). Nevertheless, if a reduced food intake were the only cause of weight loss, then supplemental nutritional support should completely reverse cachexia. Yet even enteral or parenteral hyperalimentation is unable to totally reverse the altered body composition of cachectic cancer patients (Nixon et al., 1981; Cohn et al., 1982). Furthermore, the cachectic lung cancer patients had lost protein almost entirely from the skeletal muscle protein mass (Figure 5.1). If they had lost weight simply due to starvation, protein would also have been lost from the non-muscle protein compartment (Heymsfield and McManus, 1985).

An alternative explanation to account for a negative energy balance and weight loss might be that the energy expenditure of cancer patients is increased. It has been suggested that in cancer patients whole body protein turnover is elevated and that the energy cost of this leads to a rise in host energy expenditure (Jeevanandam et al., 1984; Eden et al., 1984). Several investigators have measured either whole body protein turnover or resting energy expenditure in small heterogeneous groups of cancer patients. However, the results have been conflicting showing that whole body protein turnover may be normal (Glass, Fern and Garlick, 1983; Emery et al., 1984a) or increased (Heber et al., 1982; Jeevanandam et al., 1984) and that resting energy expenditure can be decreased, normal or increased (Knox et al., 1983). One aim of this study was to determine, in large homogenous groups of patients, whether rates of whole body protein turnover are increased and, if so, whether they are associated with an increased resting energy expenditure and hence with weight loss (See Chapter 6).

Rates of protein turnover were increased in patients with either lung or colon cancer (Chapter 6, Table 6.2.). However, there was no correlation between individual rates of whole body protein turnover and either rates of resting energy expenditure (Figure 6.1) or weight loss (Figure 6.2). Thus although whole body protein turnover would appear to be elevated in the cancer host, the energy cost of this is not sufficient to increase resting energy expenditure, nor is it necessarily associated with weight loss. It might be argued that the energy expenditure of the cancer patients had increased but that the methods which were used were unable to detect this small increment. However, the fact that weight stable cancer patients had elevated rates of whole body protein turnover indicates that for at least a proportion of cancer patients the added burden of elevated whole body protein turnover is within the limits at which energy balance can be maintained.

One of the main problems in determining which metabolic abnormalities might lead to an elevated energy expenditure is that an individuals resting energy expenditure is a composite measurement of all energy dependent metabolic processes. Thus, there are numerous variables apart from the one under study which might affect energy expenditure. Moreover, since inter-individual variation can be more than 50% (Garrow, 1985) it is unlikely that even in homogenous groups of patients a metabolic change which increases energy expenditure by 5 - 10% would be detected. Yet over a period of months a change of this magnitude would be highly significant in terms of a cumulative energy deficit and thereby weight loss.

In an attempt to overcome the problems of heterogeneity and interindividual variation two animal models of cachexia were studied (see Chapter 7). Body composition analysis showed clearly that in the case of the Walker 256 tumour model weight loss is due to a transfer of protein from the host to the tumour (Figure 7.1). However, in the MAC-16 tumour model the tumour is small and does not act simply as a nitrogen trap. Mice bearing the MAC-16 sustained a greater protein loss than was gained by the tumour (Figure 7.2). Moreover, carcass fat was reduced by about 50%. This suggests that the presence of the tumour had induced both a negative energy and protein balance. One explanation which might account for these changes could be that the energy expenditure of tumour bearing mice had increased. However, throughout the period of weight loss there was no change in the daily energy expenditure of the mice (Table 7.1). An alternative explanation could be that the food intake of the mice had decreased. However, a reduction of food intake was demonstrated only when the mice had already lost 20% of their body weight (Figure 7.4). There was no evidence that either the tumour or the development of cachexia had altered the rate of

protein synthesis in the tissues of the mice (Table 7.2).

Although the results of this study were inconclusive and thus disappointing they did raise an important factor which must be considered in future studies. The duration of weight loss is such that a small change in any process involved in the overall energy balance of the body could account for the severe weight loss observed. Thus it might be that the sensitivity of the techniques available is not sufficient to be able to detect these changes. Clearly it is necessary optimise the accuracy and precision of measurements of food intake and energy expenditure. More accurate documentation of food intake in animal experiments will principally involve keeping food scatter to a minimum. For humans, rather than a subjective recall of dietary intake, direct observation and meal portioning by a trained dietician is probably necessary. One approach to minimise intra-individual variation in daily energy expenditure would be to measure energy expenditure over several days or weeks. Recent developments in stable isotope methodology means that it is now possible to measure the energy expenditure of humans or small animals over a period of several days or weeks (Prentice et al., 1985). Since patients or animals will not be restricted by the techniques of indirect calorimetry this may well give a more accurate reflection of the natural history of the development of their negative energy balance. Moreover, the limited participation of the patients required for this technique may allow sequential studies on the same individuals and thereby cut down inter- individual variation.

Nutritional support has been tested extensively as a method of reversing the nutritional problems of cancer patients. It was hoped

that this might improve patients tolerance to conventional antineoplastic therapy and also prolong survival (Copeland, Daly and Dudrick, 1977). However, standard enteral or parenteral hyperalimentation is unable to reverse completely the abnormal body composition of cachectic cancer patients. In particular, replenishment of the patients protein mass is difficult to achieve (Cohn et al., 1982). One explanation for this might be that nutritional support leads to an acceleration of tumour growth rate. Certainly, an increased rate of tumour growth has been demonstrated in parenterally fed tumour-bearing rats (Popp, Morrison and Brennan, 1981; Cameron, 1981). Furthermore, it is now evident that partial repletion of host tissues will not improve the outcome of only partially effective chemotherapy or radiotherapy (Brennan, 1981). One of the aims of this thesis was to evaluate two novel approaches which might be used to reverse the host-tumour imbalance in cachexia.

Systemic ketosis was evaluated as a therapy in cancer cachexia because several tumour cell lines have been shown to be unable to use fat or ketone bodies as energy substrates (Tisdale and Brennan, 1983) and rely predominantly on glucose for energy production (see Demetrakopoulos and Brennan, 1982). Thus ketone bodies might act as a 'host specific' energy substrate (Tisdale and Brennan, 1983). In addition, the limited supply of glucose in the semistarving cancer host is thought to be augmented by its synthesis de novo from amino acids derived from the breakdown of muscle protein (Gold, 1968). It has been proposed that ketone bodies might directly inhibit muscle proteolysis (Sherwin, Hendler and Felig, 1975) and thereby might reduce the supply of amino acids for accelerated gluconeogenesis in the cachectic cancer host (Williams and Matthaeis, 1981). Thus, host muscle would be preserved and glucose supply to the tumour might be reduced. Moreover,

since ketone bodies would not be available as energy substrates for the tumour there should be no stimulation of tumour growth on refeeding. This hypothesis was tested by examining tumour growth rate and host nitrogen losses in rats bearing the Walker 256 tumour fed either a normal or ketogenic diet (see Chapter 8). The effects of systemic ketosis on the nitrogen metabolism of cachectic patients was also evaluated (see Chapter 9).

It was shown that the Walker 256 tumour could probably not utilize ketone bodies as a major energy substrate since the latter lacked one of the key mitochondrial enzymes required for the metabolism of either acetoacetate or 3-hydroxybutyrate (Table 8.1). However, systemic ketosis had no effect on tumour growth rate or host nitrogen losses. Moreover, the reduction in blood glucose concentration observed in ketotic non-tumour bearing rats was not observed in the tumour-bearing rats (Table 8.2). This lack of effect of systemic ketosis on blood glucose concentration may partly explain why ketosis did not inhibit either tumour growth rate or the development of cachexia.

Although the blood glucose concentration of patients with cancer cachexia was slightly reduced following the induction of ketosis (Table 9.4) there was no effect on either whole body protein turnover (Table 9.8) or nitrogen balance (Table 9.7). This agrees with the findings of several recent studies which have shown no direct effect of infused 3-hydroxybutyrate on the protein metabolism of humans (Miles et al., 1983; Takala, Peltola and Kirvela, 1985). Thus, although systemic ketosis might be used as a method of providing extra energy to the host whilst not increasing glucose supply to the tumour, the lack of effect on host protein metabolism or tumour growth rate suggests that ketosis

on its own would not markedly change the host-tumour imbalance of cachexia.

The final aim of this thesis was to determine whether tumour energy metabolism could be used as a target for selective and therefore non-toxic systemic anticancer therapy (See Chapter 10). The successful elimination of the tumour is probably the most promising approach to the treatment of cachexia. Ever since the original observations of Warburg (1932) evidence has been accumulating which suggests that the energy metabolism of tumour cells is different from that of normal cells (see Pedersen, 1978). However, not all tumour cells have the same abnormalities and there is a broad spectrum of aberrations (Weber, 1977). Various isolated attempts have been made to make use of these abnormalities to selectively inhibit either oxidative phosphorylation or glycolysis within tumour cells (see Demetrakopoulos and Brennan, 1982). However, it has been shown that some tumour cells in vitro obtain as much ATP from glycolysis as from the much more efficient process of oxidative phosphorylation (Nakashoma, Paggi and Pedersen, 1984). Thus inhibiting only one process may not reduce intracellular ATP levels to the extent that tumour cell viability is compromised.

In an attempt to selectively kill tumour cells in vivo Rhodamine 6G (a potent inhibitor of oxidative phosphorylation) was administered to fasted tumour bearing rats at the same time as hypoglycaemia was induced with 3-mercaptopicolinic acid. The latter compound inhibits phosphoenolpyruvate carboxykinase which is a key enzyme in the gluconeogenic pathway. Neither Rhodamine 6G or the period of hypoglycaemia reduced tumour growth rate. However, the combination which was designed to inhibit both glycolysis and oxidative phosphorylation reduced final tumour weight by 50% and was non toxic to the host (Figure 10.5).

It is therefore apparent that tumour energy metabolism may be a useful target for non-toxic anticancer therapy. In particular, simultaneous manipulation of both glycolysis and oxidative phosphorylation may enhance anti-tumour selectivity. Future work should evaluate the ability of various inhibitors of glycolysis and oxidative phosphorylation to selectively inhibit these processes within tumour cells. In addition, further knowledge about the particular nutritional requirements of both normal and neoplastic tissues will help in the design of nutritional perturbations which might be selectively antineoplastic.

Clearly the best way to cure cachexia is to cure the cancer. Thus, selective manipulation of the tumour energy metabolism may be a method of significantly improving the survival of cachectic cancer patients. Meanwhile, continuing the investigation into the mechanisms of weight loss and the mediators of these effects in cancer patients may provide the means to redress more effectively their nutritional problems and thereby reduce the morbidity and mortality resulting from neoplastic disease.

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