AN INVESTIGATION OF THE PROTEIN METABOLISM IN HEALTHY SUBJECTS AND WEIGHT-LOSING CANCER PATIENTS

by

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F.I.M.L.S.

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DECLARATION

I declare that the work presented in this thesis has been carried out solely by me, except where indicated below. The sample preparation and isotope analysis was performed by myself.

In 1986, I was working in Dr Preston's laboratory carrying out ^{15}N isotopic analysis of urinary end-product samples (urea and ammonia) generated by protein turnover studies (Fearon et al., 1988) when the first prototype commercial Continuous Flow-Isotope Ratio Mass Spectrometer was installed. Since then I have been developing, with Dr Preston, the new technique of Continuous Flow-Isotope Ratio Mass Spectrometry. This work has resulted in the publication of a number of methodological papers (Preston and McMillan, 1988; McMillan, Preston and Taggart, 1989; Preston and McMillan, 1990). During this time, I have been largely responsible for the application of such methods for stable isotope measurement to clinical studies. These studies, in addition to the work described in this thesis, have included measurement of palmitate turnover in normal subjects and weight-losing cancer patients (Selberg et al., 1990); measurement of whole body protein kinetics in normal subjects and weight-losing cancer patients (Fearon et al., 1990); measurement of whole body protein kinetics before and after surgery (Taggart et al., 1991a); measurement of energy expenditure before and after surgery (Taggart et al., 1991b); measurement of energy expenditure in patients with ischaemic heart disease (Taggart et al., 1991c). The contribution of this work to the development and refinement of Continuous Flow-Isotope Ratio Mass Spectrometry has been acknowledged (Barrie et al., 1989).

The study of protein synthesis in normal subjects was performed jointly with Mr K.C.H. Fearon and Dr T. Preston. This work was presented

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DEDICATION

To my wife, Fiona.

SUMMARY

The majority of cancer patients with progressive disease lose weight and a proportion become emaciated to the point where they appear to die of starvation. This complex metabolic syndrome characterised clinically by progressive involuntary weight loss is termed cancer cachexia. The importance of this cachexia has been recognised for at least 50 years and it has been reported that it is responsible for between 10 and 20% of all cancer deaths. Furthermore, more than 50% of cancer deaths are attributable to sepsis and there remains the complex relationship between nutritional depletion and a propensity to infection. The mechanisms which underlie cancer cachexia are poorly understood.

In the last decade, there have been a number of studies which have demonstrated a significant increase in the rate of whole body protein synthesis of weight-losing cancer patients. Since the energy cost of whole body protein synthesis is thought to be approximately 20% of resting energy expenditure it has been postulated that such increased whole body protein synthesis, in the presence of reduced food intake, may contribute to a continuing negative energy balance, and thus weight loss, in these patients. However, it is not known which tissues in the body contribute to elevated protein flux in cancer patients.

The main aim of this thesis was to develop suitable methodology to allow investigation of the basis of the increased whole body protein synthesis in cancer patients. It has been estimated from work on animals that liver and skeletal muscle make up approximately 60% of daily protein synthesis in the whole body. Therefore, following a review of literature methods were developed, using stable isotope-labelled amino acids, to measure simultaneously not only whole body protein synthetic rate but also protein synthetic rates in liver and skeletal muscle in man.

Protein synthetic rate measurements involve detection of small changes in isotopic enrichment requiring the use of a low resolution mass spectrometer. It is essential to purify samples for presentation to the mass spectrometer. Therefore, sample preparation protocols were developed to allow separation of leucine and glycine, in a pure form, from complex samples such as plasma, intracellular fluid and hydrolysed protein. Sample preparation methods were developed to enable measurement of ^{15}N enrichment in urinary ammonia, plasma glycine, homogenate free glycine and glycine from tissue protein hydrolysate. Sample preparation methods were also developed to enable measurement of ^{13}C enrichment in breath CO_2, plasma leucine and leucine from tissue protein hydrolysate. Continuous Flow-Isotope Ratio Mass Spectrometry was used for the isotope enrichment measurements presented in this thesis, rather than the conventional approach (requiring both a Gas Chromatography-Nass Spectrometer and Isotope Ratio Mass Spectrometer). This permitted the analysis of all samples with a single mass spectrometer.

To obtain normal values for whole body and tissue protein synthetic rates, a group of weight-stable normal subjects (n=6) undergoing elective cholecystectomy were studied. Two labelled amino acids ([^{13}C]leucine and [^{15}N]glycine) were used to assess which in the context of the present study would give the most reliable results. The precision and accuracy of the measurement of [^{13}C]leucine enrichment was poorer than that of [^{15}N]glycine. Furthermore, [^{15}N]glycine appeared to give the most reliable results and therefore was used in the study of a group of (n=6) weight-losing cancer patients.

In comparison with the control group the mean rate of whole body protein synthesis in the cancer patients was increased by 71%. However, when individual tissues were studied, non-export liver protein synthesis

was reduced by 32% whereas skeletal muscle protein synthesis was increased by 86% in the cancer patients. Therefore, much of the increase in whole body protein synthesis in weight-losing cancer patients appears to be due to an increase in skeletal muscle protein synthesis. There was no significant difference in resting energy expenditure between the two groups. Furthermore, the increase in skeletal muscle protein synthesis conflicts with previous reported results and other indirect measurements in this group of patients. These results posed two questions. Firstly, does the incorporation of ^{15}N into a metabolic end-product (ammonia) reflect the change in whole body protein synthesis in weight-losing cancer patients? Secondly, what is the basis of the increased incorporation of [^{15}N]glycine into the muscle of the weight-losing cancer patients? One explanation, discussed in detail, is that in the cancer patients increased amounts of glycine-rich proteins are synthesised as part of the inflammatory response and bring about the apparent increase in whole body protein synthesis and increased [^{15}N]glycine enrichment in the skeletal muscle.

It is concluded, firstly, that the analytical approach developed in this thesis was satisfactory for use in [^{15}N]glycine tracer studies. Secondly, that the data presented draws attention to the limitations of extrapolating the kinetics of a single labelled amino acid to the rate of synthesis of protein either in individual tissues or in the whole body. A variety of mechanisms and mediators are discussed which might account for the altered tracer kinetics observed in the weight-losing cancer patient.

CHAPTER 1: INTRODUCTION AND AIMS

1.1 Protein metabolism in man

Proteins play a crucial role in virtually all biological processes (Stryer, 1988). Peptides and proteins have many functions within the human body, notably as enzymes responsible for metabolic processes including those involved in making energy available, as structural proteins of the skin (keratin and collagen), connective tissues, tendons and bone (collagen) and muscle (actin and myosin), as hormones (such as insulin) and as antibodies. In addition proteins are used in the transport of substances in the blood (haemoglobin and many of the plasma proteins) and in transcellular and intracellular transport processes. Protein can also serve as a critical reserve of metabolic fuel that is depleted during progressive malnutrition. The fundamental units from which all proteins are constructed are amino acids. Many proteins have additional non-protein components as integral parts of their molecules. Examples of such proteins are haemoglobin and the cytochromes (metalloproteins), glycoproteins, and lipoproteins. Proteins are synthesised from a pool of 20 different amino acids, but some proteins contain additional amino acids which are formed from the modification of one of the original 20 after the protein chain has been synthesised. The wide variety of functions of peptides and proteins is matched by the wide variety of physical and chemical characteristics. For example their molecular weights vary from 5000 daltons to several million, their shapes vary from globular to linear and their surfaces are capable of binding a wide variety of substances.

The ordered structure of protein molecules is critical to their biological function. Maintenance of this function is achieved by resynthesis and consequently proteins have a finite lifespan and are eventually

degraded to their constituent amino acids, to be replaced by newly synthesised proteins. Therefore there is a persistent need for their resynthesis (Reeds, Fuller and Nicholson, 1985).

In contrast to the ability of the body to store moderate amounts of carbohydrate and large amounts of fat, the tissues have relatively little capacity for storing amino acids or protein. Therefore efficient utilisation of amino acids from the diet or protein breakdown is necessary to maintain body function. After a protein meal almost all of the absorbed amino acids are removed from the blood by the liver and skeletal muscle so that the plasma concentration of amino acid nitrogen rises only slightly (Jeejeebhoy, 1988). Similarly, amino acids given into the systemic circulation disappear rapidly from the blood. Amino acids derived from food and the breakdown of tissue proteins form a 'metabolic pool' in the blood and tissues for the synthesis of proteins and the oxidation to CO_2 and urea. This pool serves as a source of amino acids some of which are used to build nitrogenous substances such as proteins, while the rest is degraded and the nitrogen excreted, mostly as urea. Not only do amino acids from all sources form a common pool but the amino groups of one amino acid may be transferred to other amino acids by transamination.

The labile nature of body proteins was demonstrated in one of the first studies of intermediary metabolism using stable isotopes (Schoenheimer, Ratner and Rittenberg, 1939). The term turnover has been used to describe the process of renewal or replacement of a substance. In this work protein turnover is considered to be a mass of protein (either a single protein or a mixture) which exchanges with a pool of free amino acids. The turnover is determined by the balance of synthesis and breakdown of this protein mass. In general, structural proteins have a large

pool size and low turnover rate whereas metabolic proteins have a small pool size and rapid rate of turnover.

The constant and considerable breakdown and resynthesis of most body proteins in the adult and even higher rates of both synthesis and breakdown during growth in the young are now well recognised (Waterlow, Garlick and Millward, 1978a). It is also well established that these rates of turnover vary from tissue to tissue and the pattern of contribution made by different organs and tissues to whole body protein turnover changes during development and as the organ ages (Waterlow Garlick and Millward, 1978b). Furthermore, synthesis and breakdown rates also change with alterations in diet, especially in response to altered protein intakes (Waterlow and Jackson, 1981).

The turnover of body proteins is accompanied by an extensive reutilisation of amino acids for the formation of new proteins. 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, 1978c). However, this process is not completely efficient, so that some amino acids are lost by oxidative catabolism (Waterlow and Stephen, 1966). Thus a state of dynamic protein turnover is expensive in terms of energy and to a lesser extent, amino acids.

It is clear that simple nitrogen balance studies in man cannot address questions about protein dynamics in health and disease since alterations in intensity, quality and the distribution of tissue and organ protein metabolism are not revealed. Also these studies do not provide any insight into the changes in protein synthesis and/or breakdown which determine nitrogen balance. Therefore, protein turnover measurements can be of

considerable value. In order to measure protein turnover it is necessary to use labelled amino acids as tracers.

1.2 Turnover measurements using isotope tracers

The use of stable isotopes for tracer studies began in the 1930's at Columbia University, where in early studies, Schoenheimer and Rittenberg (1935b) used the stable isotope of hydrogen (deuterium) to study fat metabolism in mice. Shortly after this they used [^{15}N]glycine to demonstrate the dynamic nature of body protein in man (Schoenheimer, Ratner and Rittenberg, 1939). When ^{2}H, ^{15}N, and ^{13}C became more readily available, isotope labelling techniques were applied to metabolism studies in many laboratories. Of particular importance were the pioneer studies with ^{13}C on CO_2 fixation and various aspects of carbohydrate metabolism in plants and animals (Rittenberg and Waelsch, 1940; Wood et al., 1940; Rittenberg and Foster, 1940). From this and other work the concept of the metabolic pool was developed (Rittenberg, 1949).

However, the introduction of scintillation counting and the availability of a wide variety of radioactive tracers resulted in radioactive tracers (^{14}C, ^{3}H) being used for most metabolic pathway studies in the 1950's and 1960's. Throughout the period 1950-1970, when radioactive tracer administration was most active, nutritionists remained the principal users of stable isotope tracers for clinical investigation. There were several reasons; (a) the dynamics of substrate interactions with body fuel stores were first revealed with stable isotope tracers (Schoenheimer and Rittenberg, 1935a); (b) deuterium oxide dilution had proven to be a safe and reliable means of estimating total body water for body composition analysis; (c) radio-tracer use was prohibited in populations of malnourished children where urgent questions of pathophysiology and therapy awaited

quantitative data; (d) nitrogen and oxygen have no radioactive equivalents of sufficiently long half-life to undertake metabolic studies in man. Even so, limited availability of compounds labelled with stable nuclides and the more restrictive analytical requirements compared with the equivalent radio-tracer measurement, limited growth of stable isotope tracer applications in human investigations over this period.

In the last 15 years this situation has changed considerably. Stable nuclides separated at various national laboratories have been available in quantity. In addition, the coincident microelectronics 'revolution' has helped make available a range of computer controlled analytical systems capable of sensitive, precise quantitation of stable isotope tracers in biological materials. Also over this period, the scientific drive for mapping metabolic pathways decreased, as more and more pathways were elucidated. Much of this work was done using radioisotope-labelled substrates. The key question for many investigators in this field has changed from what were the intermediate substrates of the metabolic pathway to what amounts of these substrates were being consumed in the pathway (ie from 'what goes' to 'how much'?; Kascer and Burns, 1973). The need to answer such questions in man has increased demand for stable isotopes. Lastly, investigators have realised that stable isotope tracer techniques offer advantages over radio-isotopes (in addition to the avoidance of radiological hazard). These advantages include the ability to measure simultaneously substrate content and isotope enrichment (Bougneres and Bier, 1982); the capacity to determine the intramolecular location of the tracer labels (Matthews et al., 1981a); the ability to study the same individual repeatedly in order to assess intrasubject variability (Fern, Garlick and Waterlow, 1985a), natural progression, or the effects of therapeutic intervention (Conley et al., 1980); the ability to use several

isotope tracers simultaneously (Kahlan et al., 1980); and the better sensitivity of the stable isotope technique, when the amount of tracer is restricted on ethical grounds and is subjected to a large dilution, e.g., incorporation of a label into tissue.

1.3 Measurement of stable isotopes

Protein metabolism studies using ^{15}N or ^{13}C-labelled amino acids as tracers can generate considerable numbers of samples for analysis. Therefore, appropriate instrumentation for the measurement of these isotopes is an important consideration in undertaking such studies. Until recently isotope analysis of samples from such studies required the use of two mass spectrometers, Isotope Ratio Mass Spectrometry for analysis of the relatively large concentrations of low enrichment metabolic end-product and Gas Chromatography-Mass Spectrometry for analysis of the labelled substrate usually present at high enrichment but low concentration. The recent development of Continuous Flow-Isotope Ratio Mass Spectrometry, combined with cation exchange High Performance Liquid Chromatography, developed as part of this thesis (Chapters 3 and 4), offers an alternative to such conventional approaches (Preston and Owens, 1983,1985; Preston and McMillan, 1988).

1.4 Protein turnover measurements

In the majority of tracer studies in man, it is now considered necessary to use stable isotopes. In protein turnover studies not only is there a choice of stable isotopes, (mainly ^{13}C or ^{15}N), but also a variety of amino acids to act as tracers. Alanine, glycine, leucine, lysine, phenylalanine, and tyrosine have all been used in such studies, however most measurements have been made with [^{15}N]glycine or L-[1-^{13}C]leucine.

Glycine was the first amino acid used to estimate whole body protein turnover as it was readily purified and has no isomers. Its use has been extensive, but in the last 10 years, L-[^{13}C]leucine has been used increasingly. This has reflected a move away from end-product analysis to precursor analysis. The perceived advantages of each substrate are discussed in detail in Chapter 2.

1.5 Cancer cachexia

1.5.1 Weight loss in cancer

The majority of patients with progressive 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 complex metabolic syndrome characterised clinically by progressive weight loss is termed cancer cachexia. The word cachexia is derived from two greek words, 'kakos' and 'hexis' meaning 'poor condition' and is descriptive of any disease that results in host tissue wasting. The importance of cancer cachexia has long been recognised. Warren (1932), in a post mortem study of 500 cancer patients concluded that 22% of deaths were directly attributable to cachexia and this was the single most common cause of death in cancer patients. More recent studies (Inagaki, Rodriguez and Bodey, 1974) have suggested that this figure is closer to 10%. However, more than 50% of cancer deaths are attributable to sepsis and there remains the complex relationship between nutritional depletion and a propensity to infection. Furthermore, patients with a marked weight loss have a poor tolerance of vigorous antineoplastic therapy with lower response rates and an increased risk of suppression of cell-mediated immunity as compared with weight

stable cancer patients. Hypoalbuminaemia associated with the cachetic patient is significantly correlated with the rate of post-operative complications (Daly and Thom, 1988). Given such nutritionally associated morbidity and mortality there is an urgent need to correct cachexia in cancer patients. The obvious treatment is to remove the tumour, but this is not always possible and therefore it is necessary to understand the mechanism of weight loss and try to plan alternative forms of treatment.

The pathogenesis of weight loss in cancer is not clearly understood. Weight loss does not correlate with the type of cancer or with the site or number of metastases (Costa and Donaldson, 1979). Moreover weight loss is not the result of tumour growth per se, since it is not induced by all tumours (Bozzetti et al., 1982). In general, loss or gain of body mass is due to an imbalance between energy intake and energy expenditure. A negative energy balance could be due to decreased food intake, an increased energy expenditure, or a combination of these two. Although anorexia is often a prominent symptom in wasted cancer patients, the weight lost by patients is often in excess of that expected from their documented reduction in food intake (Costa et al., 1981). This has led to suggestions that there must be a metabolic disorder contributing to the development of weight loss in cancer patients (Theologides, 1979). There are numerous reported abnormalities of intermediary metabolism in cancer-bearing animals and man involving glucose, protein, fat and energy metabolism (Kern and Norton, 1988). However, it is not clear which of these abnormalities contributes to weight loss. The persistent failure of cachetic patients to gain weight in the form of useful, metabolically active body cell mass despite seemingly adequate nutritional support may also be due to these widespread metabolic abnormalities.

1.5.2 Body composition in cancer cachexia

In order to understand the mechanisms of weight loss in cancer cachexia it is necessary to determine the nature and extent of tissue loss. Several studies over the years have attempted to define the composition of tissue loss. A study by Cohn and coworkers (1981) of cancer patients with different tumour types, in which total body nitrogen, potassium and water were measured, suggested that weight loss reflects primarily the loss of fat and muscle tissue. Furthermore, they reported that there was no change in the amount of non-muscle tissue (including visceral tissue) with progressive weight loss in the cancer patients. Preston and coworkers (1987), using neutron activation analysis to measure total body minerals and nitrogen and whole body counting to measure total body potassium, derived muscle and non-muscle protein mass by compartmental analysis. They studied body composition in a group of cachetic lung cancer patients who had lost 30% of their pre-illness weight and compared them with a group of control patients who were matched for the age, sex, height and pre-illness weight of the cancer group. The most dramatic alteration in body composition associated with the development of cachexia was the loss of greater than 80% of body fat. They concluded that since fat is the principal energy store of the body, the patients had been in a prolonged, severe, negative energy balance. The other notable finding was a 75% reduction of skeletal muscle protein mass and this accounted for almost all of the net negative nitrogen balance. In accord with other studies they reported an expansion of the extracellular water space with relative preservation of the non-muscle protein mass. Such body composition analysis seems to support the finding that weight losing cancer patients show marked muscle wasting with little change in liver, spleen, kidney and heart weights (Heymsfield and McManus, 1985). This contrasts with

simple starvation where the weight of these tissues and organs fall in parallel with that of the whole body (Heymsfield and McManus, 1985).

1.5.3 Energy metabolism in cancer cachexia

Studies of energy expenditure in cancer cachexia have shown conflicting results. Recent studies using indirect calorimetry have shown either no elevation or minimal elevation in metabolic rates in some cancer patients (Burke,Bryson and Kark, 1980; Macfie et al., 1982; Hansell et al., 1986). In contrast, others have shown that specific tumour types may be accompanied by a hypermetabolic state. Dempsey et al (1984, 1986) have shown by indirect calorimetry that gastric cancer patients have elevated energy expenditure. Peacock et al (1987) studied a homogenous group of sarcoma patients with indirect calorimetry and ^{40}K analysis of body composition. When compared to age and sex matched controls, sarcoma patients had significantly elevated resting energy expenditure, and a significantly decreased body cell mass. These patients did not have overt anorexia or weight loss. This study in humans confirms the finding of earlier studies with animal models of cancer cachexia that loss of host tissue begins early, at low tumour burdens, and precedes clinical evidence of weight loss.

Such contradictory findings in relation to the energy expenditure of patients with malignant disease may be partly due to the larger spectrum of energy expenditure in cancer compared to the normal population (Knox et al., 1983), reflecting the diverse nature of the effects of cancer on host energy metabolism. Furthermore, although results of energy expenditure studies vary, an important finding is that the metabolic rate fails to adjust to changes in food intake. Even small increases in metabolic rate must be met by equivalent increases in caloric intake, or persistent weight loss will

ensue. However, interpretation of such studies must be made in the light of the technical difficulties with measuring food intake accurately.

1.5.4 Whole body protein metabolism in cancer cachexia

Whole body protein turnover has been estimated to account for between 10% and 20% of resting 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 and Jackson, 1981). It has been suggested that this adaptive mechanism might be impaired in patients with malignant disease (Brennan, 1981). Such adaptation is essential if the host is to minimise fat and protein losses in the presence of a reduced food intake.

In an attempt to assess specifically the effects of the tumour, Glass, Fern and Garlick (1983), using oral doses of [^{15}N]glycine, measured rates of whole body protein turnover synthesis and breakdown in 11 patients with colorectal cancer before and after surgery. They did not find any significant changes in protein metabolism when comparing patients before and 12 weeks after tumour resection. They concluded that the presence of the primary tumour did not alter the overall rate of protein metabolism. However, the patients in this study had no evidence of protein calorie malnutrition and were at an early stage of the disease. In contrast, Eden and co-workers (1984) demonstrated that whole body protein turnover was increased in patients with cancer (as was resting energy expenditure) and concluded that these changes had contributed to the loss of body fat (energy) and protein (amino acid) reserves. Other studies have confirmed that whole body protein turnover is indeed elevated in patients with cancer (Norton, Stein and Brennan, 1981; Heber et al., 1982; Jeevanandam et al.,

1984; Fearon et al., 1988). As with energy expenditure measurements in these patients there is a broader range of whole body protein turnover compared with controls (Fearon et al., 1988) (Figure 1.1).

In one of the few studies to measure resting energy and whole body protein turnover simultaneously, a corresponding increase in energy expenditure was not observed in weight-losing cancer patients with increased whole body protein turnover (Fearon et al., 1988). This observation has recently been confirmed in weight stable cancer patients who had increased whole body protein turnover but no increase in resting energy expenditure (Melville et al., 1990). There could be several reasons for this apparent contradiction. Firstly, that the measurement of energy expenditure by indirect calorimetry is insufficiently sensitive to detect the small increase in energy expenditure due to increased whole body protein turnover. Secondly, that since resting energy expenditure is the sum of all energy dependent processes in the resting, postabsorptive individual, other energy requiring processes are reduced to compensate for the energy cost of increased protein turnover. Lastly, although patients with cancer may have an elevated rate of tracer flux, this might represent regional changes in amino acid flux rather than a uniformly elevated rate in the whole body. If such regional changes are responsible for an increased amino acid flux (giving an apparent increased whole body protein turnover) then it is likely that the liver and muscle compartments are involved since together these are thought to account for approximately 60% of whole body protein turnover.

1.5.5 Tissue protein metabolism in cancer cachexia

To obtain greater insight into the mechanism of altered protein metabolism in the cancer host several workers have attempted to measure rates of protein synthesis in skeletal muscle and liver. This work on rates of protein synthesis in different tissues is in its infancy, particularly in man. Not all tissues and organs have been studied, and the results are in general inaccurate since they take no account of the anatomical heterogeniety of tissues eg in the liver only about two thirds of the cells are parenchymatous cells. Moreover, in the gut, pancreas and liver an allowance has to be made for a substantial production of export protein if a complete picture of the activity of protein synthesis is to be obtained.

Skeletal muscle protein metabolism; Skeletal muscle accounts for approximately 40% of body weight in man and is thought to be responsible for approximately 40% of whole body protein synthesis. This tissue therefore has a major role in the protein metabolism of the whole body (Waterlow, Garlick and Millward, 1978b). In a study of human rectus abdominis muscle from both cancer patients and age matched controls Lundholm and coworkers (1976) reported decreased skeletal muscle protein synthesis (as measured by decreased incorporation of ^{14}C-leucine into muscle protein in vitro). It must be considered however, that such in vitro measurements may not be physiologically meaningful. Nevertheless, studies of 3-methylhistidine release (a marker of muscle breakdown) from skeletal muscle in cancer patients have also suggested that protein is lost due to decreased synthesis rather increased breakdown (Lundholm et al., 1982). These findings are in agreement with decreased skeletal muscle protein synthesis rates in animal studies in a variety of diseases characterised by malnutrition and wasting (Svaninger et al, 1983), where protein synthesis (rather than increased breakdown) is the main regulator

of muscle protein turnover (Rennie et al., 1983). However, several animal studies suggest that protein breakdown is abnormally elevated in tumour bearing host muscle. Urinary 3-methylhistidine (primarily a skeletal muscle breakdown product) has been shown to be significantly greater in rats bearing a sarcoma than in control rats (Norton et al., 1981). Emery and coworkers, (1984b) reported an 80% increase in the intramuscular free 3-methylhistidine of tumour bearing mice. Thus, it appears that tumour bearing animals may lose skeletal muscle protein either by a decrease in protein synthesis or an elevation in muscle protein breakdown or a combination of the two.

Liver protein metabolism; The liver has a central function in energy and substrate metabolism and although the liver represents only 2% of body weight it is thought to account for 20% of whole body protein synthesis (Waterlow, Garlick and Millward, 1978b). The normal liver is known to have a major synthetic capacity which is divided equally between the production of export and non-export proteins.

Studies of the cachetic effects in the tumour-bearing state on liver protein synthesis have produced paradoxical results. The consistent finding of hypoalbuminaemia in cachetic cancer patients has been assumed by clinicians to be the result of decreased hepatic synthesis of albumin (Waldmann, Trier and Fallow, 1963). However, Karlberg, Kern and Fischer (1983) using sarcoma-bearing rats determined that rates of albumin synthesis were increased. The low serum albumin levels were a result of increased albumin degradation and expansion of the intravascular fluid space.

Synthetic rates of total protein in hepatocytes isolated from tumour-bearing animals have been shown to be twice that of control hepatocytes, with the increase proportional to the tumour burden (Warren,

Jeevanandam and Brennan, 1987, 1985). Synthesis of both secretory and structural proteins was increased and because no net accrual of protein occurred in the livers of tumour-bearing rats, the protein degradation rate was assumed to be increased as well. Little work has been done specifically on fixed or structural liver protein synthesis either in man or animals and therefore their relative contribution to the apparently increased total liver protein synthesis in cancer cachexia is not known.

In summary, work on tumour-bearing animals suggests that fractional synthetic rates in liver are increased but that protein synthesis is decreased in skeletal muscle, the overall effect being an increase in whole body protein turnover. However, differences in the tumour type and the cachexia produced together with different methods of measuring protein synthesis make interpretation and comparison of results difficult. Observations on the rate of tissue protein synthesis in man are limited and the lack of in vivo data on both liver (fixed and export) and skeletal muscle means that the changes in tissue protein metabolism associated with cancer cachexia are not clearly understood.

1.6 Aims of thesis

1. To develop methods whereby whole body and tissue protein fractional synthetic rates can be measured using a single mass spectrometer.
2. To establish a clinical protocol to measure simultaneously resting energy expenditure and whole body, fixed hepatic and skeletal muscle protein synthesis, in man.
3. To use this protocol to compare the protein and energy metabolism in normal subjects with those in weight-losing cancer patients.

1.7 Plan of thesis

In Chapter 2 techniques for measuring whole body protein turnover and tissue protein synthesis rates in man are discussed. Methods developed for the enrichment measurement of precursor and end-product samples generated from the infusion of stable isotope labelled amino acids are described in Chapters 3 and 4. Methods for the measurement of other biochemical parameters and resting energy expenditure are described in Chapter 3. Validation of the study protocol is described in Chapter 4. A study in which resting energy expenditure and whole body, liver and skeletal muscle protein synthesis were simultaneously measured in a group of healthy subjects is described in Chapter 5. In Chapter 6 a second study is described in which the same parameters were measured in a group of weight-losing cancer patients. The results from these studies are discussed in Chapter 7. In Chapter 8 the aims of the thesis are discussed.

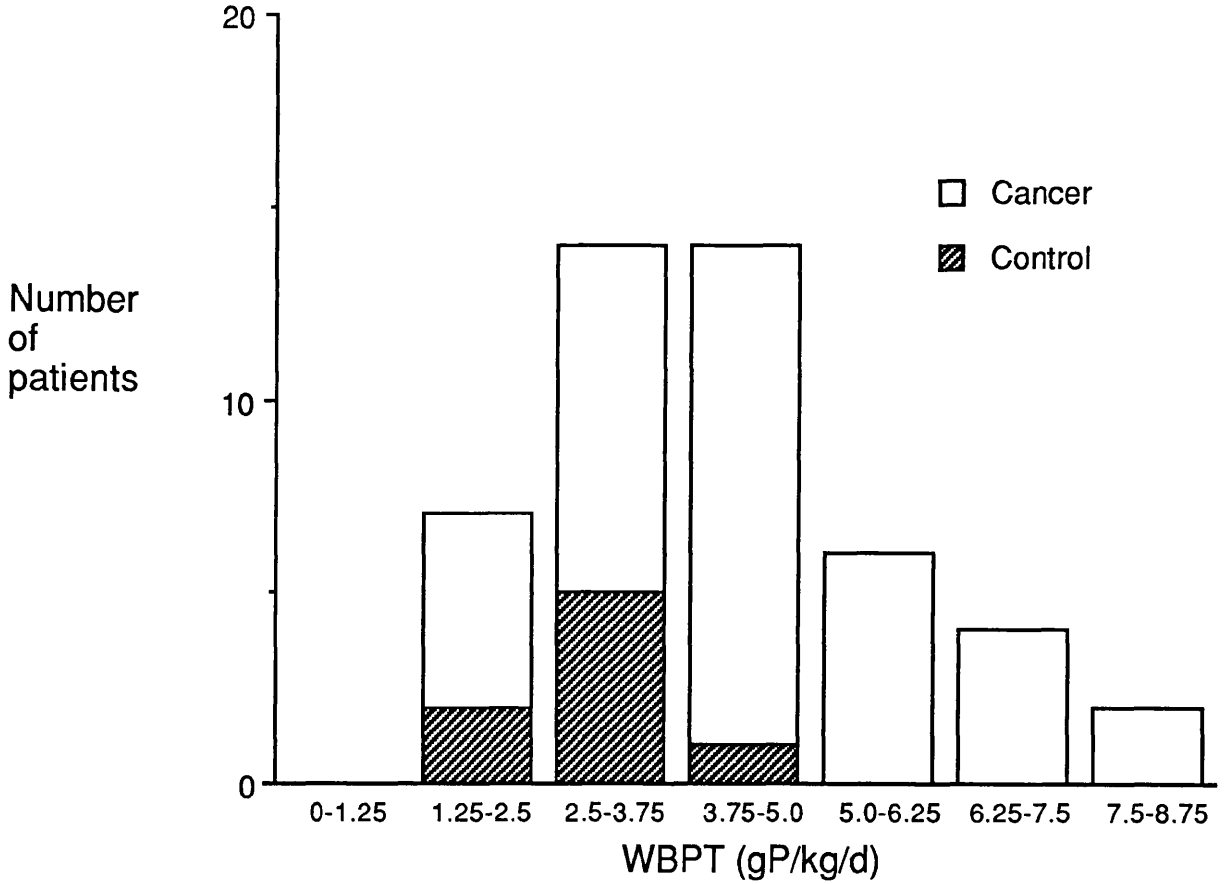


Figure 1.1 The distribution of measured whole body protein turnover (WBPT) in "normals" and cancer patients (from Fearon et al., 1988).

CHAPTER 2: TECHNIQUES FOR MEASURING WHOLE BODY PROTEIN TURNOVER AND TISSUE PROTEIN SYNTHESIS RATES IN MAN

2.1 Introduction

Estimation of whole body protein turnover requires the measurement of the rate of flux (or turnover) of a labelled (or tracer) amino acid. In order to calculate whole body protein turnover from the flux (or turnover) of an amino acid a metabolic model of protein metabolism is needed. A simple example of such a model, used extensively (Figure 2.1), is one in which the labelled amino acid, whether given intravenously or orally, enters a single homogeneous free amino acid pool, from which it can be oxidised, giving rise to CO_2 and urinary N, or it can be incorporated into protein. The protein of the whole body is considered as a single pool which is continually recycling amino acids back into the metabolic pool, but because of its large size, does not return the label during the course of the experiment. When the free amino acid pool is constant in size, its turnover (termed the flux, Q) is given by the following expression:

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

where S is synthesis, E is excretion, B is breakdown and I is intake (Figure 2.1). Using this model the protocol for the estimation of amino acid flux is determined either by compartmental or stochastic analysis of the data. Between 1950-1970, there was dispute as to which type of analysis might give the best estimate of amino acid turnover. The compartmental approach involves giving the labelled amino acid as a single dose and measuring the label enrichment in the metabolite at different time intervals, either in the pool into which the labelled amino acid was given or other pool(s). From the decay curve of label enrichment against time, fractional turnover rates of protein can be calculated by compartmental analysis

(Waterlow, Garlick and Millward, 1978d). The compartmental approach represents the conventional method which had been used from the start of stable isotope work. In theory, this approach should yield additional information, namely the ability to determine the pool size and the nature of the turnover of the pool. However, in practice, meaningful interpretation of such data has proven to be very difficult, very long protocols being necessary to estimate whole body protein turnover in man (Long et al., 1977) because of the need to define the decay constant of protein pools with very slow turnover rates. In contrast, the stochastic approach removes the assumption about the number of pools involved in protein turnover that is required for the compartmental approach (Waterlow, Garlick and Millward, 1978d). The stochastic method involves a continuous infusion of labelled amino acid until the tracer enrichment reaches a plateau in the chosen pool. Alternatively, a single dose and measurement of the cumulative tracer enrichment in the chosen pool (Waterlow, Golden and Garlick, 1978). Using the stochastic approach, fewer measurements are required to define the plateau enrichment than to define the decay curve of the compartmental approach. Also the stochastic method gives a better estimate when the enrichment of the label in a metabolic end-product is measured since once an isotopic equilibrium has been achieved the exact timing of the measurement should not matter.

It is now widely considered that the compartmental approach requires so many analyses that in practice the benefits are not worth the extra effort required when compared to the stochastic method. Indeed, with reference to whole body protein turnover, Waterlow, Garlick and Millward (1978d) have suggested that the compartmental approach is in error as it does not properly describe the underlying processes. In clinical studies, to enable stochastic analysis the continuous infusion has become

the most commonly used method of administering the labelled amino acid as it is convenient and gives essentially the same turnover values as single or multiple dose regimens (Garlick and Fern, 1985). In such studies a bolus dose (termed the priming dose) of the tracer is often given in conjunction with a continuous infusion to shorten the time taken to reach isotopic equilibrium in the metabolic pool. The use of a priming dose not affect the plateau isotopic enrichment value, only the time taken to reach it (Wolfe, 1984a).

Attempts to measure the rate of amino acid flux in man with stable isotopes have followed the development of the mass spectrometer. In most early studies which used stable isotopes the enrichment of the amino acid label in a metabolic end-product was used to derive turnover rates (end-product methods) rather than the enrichment of the amino acid in the metabolic pool (precursor methods). This was due to the Isotope Ratio Mass Spectrometer being the only available instrument for measuring stable isotope enrichment and its requirement of a relatively large sample size for analysis. Furthermore, if one uses a precursor method with Isotope Ratio Mass Spectrometry, then purification of the sample from plasma or the relevant metabolic pool for enrichment analysis is complex and time consuming, and small sample sizes often necessitate isotope dilution analysis.

Gas Chromatography-Mass Spectrometry together with new automated techniques for precise analysis of specific ions (selected ion monitoring) allowed precursor methods to be carried out more easily in studies of whole body protein metabolism. Gas Chromatography-Mass Spectrometry combines the ability of the gas chromatograph to resolve components of a complex mixture with the highly selective and sensitive detection of a mass spectrometer. Therefore, separation of the labelled

tracer from a complex sample matrix (plasma, intracellular fluid) and enrichment analysis required for precursor methods became rapid and much simpler. In practical terms, there was now a choice to be made as to which approach was used for the measurement of whole body protein turnover in such studies.

2.2 Precursor methods

The constant infusion of carboxyl-labelled [^{13}C]leucine is a good example of this approach (Matthews et al., 1980). Using the model for amino acid metabolism described in the previous section, at steady state, gives the relationship

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

Where Q is the rate of leucine flux, S is the rate of leucine incorporation into protein (protein synthesis), E is the rate of leucine oxidation (or catabolism), B is the rate of leucine release from protein (protein breakdown) and I is the rate of exogenous leucine intake. The [^{13}C]leucine is infused intravenously until the isotopic enrichment of the amino acid in the plasma reaches a constant value (E_p) from which the flux can be calculated from the expression

$$Q = i / [E_i/E_p - 1]$$

Where i is the rate of infusion of [^{13}C]leucine, E_i is the enrichment of the [^{13}C]leucine infused. The plateau can be achieved more rapidly if a priming dose is given (Clague et al., 1983). At the same time the excretion of the label in respiratory CO_2 also reaches a plateau (e), from which the rate of oxidation (E) of leucine can be calculated

$$E = e / [E_p - E_i]$$

Knowledge of the dietary intake of the amino acid and body leucine content then enables the rates of whole body protein synthesis (S = Q-E) and breakdown (B = Q-I) to be estimated (Matthews et al., 1980).

There are a number of assumptions that effect the validity of this approach; firstly, it is assumed that the free amino acid pool is homogeneous and that the enrichment of leucine in a blood sample can be taken as representative of leucine at the sites of protein synthesis and leucine oxidation. However, compartmentation of the amino acid pools, both among and within tissues, has been demonstrated. For instance, it has been demonstrated that intracellular leucine enrichment is lower than that in plasma (Waterlow, Garlick and Millward, 1978d). It is therefore important to know which precursor enrichment should be used in the calculation of turnover. One way around this problem is to assume that protein synthesis and leucine oxidation take place from the same precursor pool. The rate of synthesis can then be calculated from the labelled CO_2 production (Garlick and Clugston, 1981, Golden and Waterlow, 1977). A modification of this method is to use the enrichment, in the plasma, of the transamination product of leucine, alpha ketoisocaproate (Matthews et al., 1982). The enrichment at the site of leucine oxidation is 10-30% lower than that in the plasma, and is similar to that in skeletal muscle. If one, therefore, uses alpha-ketoisocaproate to calculate leucine enrichment in the precursor pool this gives rise to higher rates of flux and oxidation than if plasma leucine enrichments are used.

A second potential source of error with labelled leucine infusion is the assessment of labelled CO_2 production. This is usually done by separate measurements of the total rate of CO_2 production and its ^{13}C enrichment. Total CO_2 production is best measured with a continuous flow through system (eg. a ventilated hood) as a Douglas bag and valve is

prone to serious error, especially with collections of a short duration (Garlick and Clugston, 1981). The main difficulty with this measurement is the apparent fixation of the isotope, resulting in recoveries of injected or infused ^{13}C-bicarbonate of less than 100%. This problem can largely be overcome if the measurement system is calibrated by infusion of labelled bicarbonate as part of the labelled leucine infusion study (Clague, Keir and Clayton, 1979; Clugston and Garlick, 1983). The other assumptions which are common to both precursor and end-product analysis are; (1) that the metabolic pool does not change in size during the period of measurement. (2) the isotope tracer is treated in the same way as the major isotope. (3) the dose of the labelled amino acid given is tracer dose and does not itself affect turnover. (4) The amino acid faithfully represents protein flux.

2.3 End-product methods

The end-product methods, with few exceptions, have used a ^{15}N-labelled amino acid as the tracer and urinary ammonia or, urea as an end-product, where it is recognised that the ^{15}N will not remain attached to one single amino acid. The ^{15}N is therefore regarded 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 nitrogen pool, their labelling can be taken as representative of that pool. An example of this approach is the continuous intravenous infusion of [^{15}N]glycine until a plateau in the labelling of ammonia or urea (S_{max}) in the urine has been achieved. This plateau value can then be used to calculate the total nitrogen flux (Q) in a way similar to that for leucine

$$Q = d / S_{max}$$

Where d is the rate of infusion of [^{15}N]glycine. The rate of amino acid oxidation (E) is given by the rate of total nitrogen excretion. Once again

knowledge of the dietary intake of nitrogen and the nitrogen composition of body protein enables the rates of whole body protein synthesis (S = Q-E) and breakdown (B = Q-I) to be estimated (Waterlow, Garlick and Millward, 1978d). Modifications have included the administration of the ^{15}N as a single dose, and the use of ammonia as the end-product rather than urea (Waterlow Golden and Garlick, 1978).

The most important assumption of the end-product method is that the fraction of the dose of isotope excreted in the chosen end-product is the same as that of unlabelled nitrogen. For this assumption to be completely satisfied, the metabolic pool should not undergo compartmentation either anatomically or metabolically. Anatomical compartmentation has been implied from rates of turnover derived from urea and ammonia after a single dose of [^{15}N]glycine (Fern and Garlick, 1981). In general the two end-products do not give the same values, the value derived from urea is higher than that from ammonia, and the difference is increased by feeding and by intravenous compared with oral adminstration of the isotope. Fern and Garlick (1981) attributed these differences to the localisation of urea synthesis in the liver and ammonia synthesis in the kidney, and have suggested a two compartment metabolic pool consisting of urea precursors (gut and liver) and ammonia precursors (peripheral tissues). Further, they suggested that the average of the two rates given by urea and ammonia (termed end-product average) would give the best estimate for whole body protein turnover. This average is unbiased towards either compartment, since its value is not affected by the route of administration of the isotope (Fern, Garlick and Waterlow, 1985b).

Metabolic compartmentation of nitrogen metabolism can be demonstrated by administration of different labelled amino acids (Fern,

Garlick and Waterlow, 1985a). Rates of protein synthesis were estimated by measurements of the excretion of ^{15}N in urea and ammonia, after both oral and intravenous administration of a number of [^{15}N]-labelled amino acids. In general, the urea and ammonia gave different values, and this difference varied greatly depending on the route of administration, thus confirming for the other amino acids the concept of anatomical compartmentation described above for glycine. It is also notable that with the other amino acids the value of the end-product average varied little with the route of administration. However end-product averages did vary greatly among the different amino acids. Metabolic compartmentation can be seen, therefore, to be an important consideration that must be allowed for when choosing a ^{15}N labelled amino acid for this type of study. Glycine, which has been used in the majority of past studies, gives rates of synthesis that are both in the middle of the range of those given by others, and are close to those given by precursor methods such as labelled leucine infusion (Fern, Garlick and Waterlow, 1985a).

The other assumptions with end-product methods are; (1) that dietary total nitrogen and glycine-N are treated in the same way as nitrogen derived from tissue breakdown. (2) [^{15}N]glycine, and any amino acid deriving ^{15}N from glycine, equilibrate through their respective pools so that their enrichment is the same at all sites of protein and end-product synthesis. (3) Glycine-N does not give rise to quantitatively significant amounts of products other than protein and excretory products and the end-products do not derive nitrogen from non-protein sources (eg. purines, creatine, hippurate etc).

Discussion as to whether precursor or end-product analysis gives the best estimate of substrate turnover has been continuous since the availability of both Gas Chromatography-Mass Spectrometry and Isotope

Ratio Mass Spectrometry instrumentation. Each type of analysis has its own advantages and disadvantages, but it appears that in recent years that there has been a move away from end-product analysis.

In summary, whether the tracer is given by a single dose or by constant infusion, precursor measurements have the advantage that with a suitable choice of tracer the duration of the experimental protocol can be relatively short e.g. 4-12h with intravenous infusion of ^{13}C-leucine. This minimises the effect of recycling. It also means that the method is more suitable for use in patients whose clinical state may be changing. However, if ^{13}C is used as a label separate mass spectrometers are required for measuring the enrichment in plasma and breath CO_2. There are two main sources of error in the precursor method. The first, is that estimates of turnover are likely to be low, because the enrichment of the tracer in the plasma is higher than that in the precursor pool. The second arises when the turnover of a single labelled amino acid is used to derive the turnover rate of body protein since it is assumed that the body protein has a particular amino acid composition. End-product measurements have the advantage in the clinical field that they are simple to carry out and the isotopic measurements are straightforward using a single mass spectrometer. The disadvantage is that, like the precursor method, there are doubts about some of the assumptions on which the method is based.

Thus, measurement of whole body protein turnover by precursor and end-product methods is based on assumptions that are not always valid. However, under normal circumstances, with a normal diet and turnover rate, the methods give similar and reasonable results. Furthermore, there appears to be no reason why such errors should affect the validity of comparative measurements. However, at the extremes of intake or when

unusual mixtures of proteins are made or disposed of, the results may be in error. It is therefore important that careful consideration is given to the design of experiments to minimise the errors which the investigator can quantify or allow for.

2.4 Tissue Protein Synthesis Measurement

Tissue protein synthesis is estimated by measuring the enrichment of the tracer incorporated into tissue protein and the isotopic enrichment of the free amino acid at the site of protein synthesis. Therefore, it is fundamental to the estimation that the source of labelled amino acids for protein synthesis is known. The measurement of the enrichment of the aminoacyl-tRNA should give a true enrichment value with which protein fractional synthetic rates can be calculated. However, this approach may be complicated by there being more than one pool of aminoacyl-tRNA at differing enrichments (Waterlow, Garlick and Millward, 1978j). Furthermore, the isolation of aminoacyl-tRNA required to make such measurements is technically difficult because of the small amount of tRNA present in tissues and its extremely rapid turnover rate. This approach has only been used in a few studies for tissue protein synthetic rate measurements (Waterlow, Garlick and Millward, 1978j). The approach which has been used extensively in such measurements is to measure the enrichment of the free amino acid in the pool thought to charge aminoacyl-tRNA. However, there has been considerable debate about which free amino acid pool is responsible. There have been a number of studies which have suggested that the source of free amino acids for protein synthesis is from the intracellular pool of the tissue (Waterlow, Garlick and Millward, 1978j). This is what would be expected since the site of protein synthesis (the ribosome) is intracellular. However, there is

also evidence that the source of particular amino acids for protein synthesis may be extracellular and these amino acids are channelled via membrane transport systems into the ribosome (Waterlow, Garlick and Millward, 1978j). It has been proposed that the source of an amino acid (intracellular or extracellular) for protein synthesis may be determined firstly by the relative concentration of the amino acid intra- and extracellularly. Secondly, by the amino acid transport mechanism involved (Waterlow, Garlick and Millward, 1978j). Therefore, it would appear that when using this approach to determine tissue protein synthetic rates it is important that both these factors and the synthetic rates calculated from intra- and extracellular amino acid enrichment are considered.

The basis for these calculations are well described for animal studies (Zak, Martin and Blough, 1979., Garlick, 1980). In animals, most tissue protein synthesis measurements have been made with radioisotopes because the specific activities of amino acids in individual proteins are easier to measure than the corresponding stable isotope enrichments and there is no limitation on biopsy size. However, in man, because of ethical considerations, stable isotope methods have been developed for measuring tissue protein synthetic rates. Two principal methods have been used to determine tissue protein synthesis in man.

2.5 Tracer dose protocol

There have been very few direct measurements of tissue protein synthesis in man, but of these most have used a continuous infusion of a labelled amino acid at a tracer dose. Halliday and McKeran (1975) in the first study in man used this approach to measure muscle protein synthesis and whole body protein turnover simultaneously. They infused an essential amino acid, L-[^{15}N]lysine for 20-30h. [^{15}N]lysine was chosen as it was

readily available and transfers only a small fraction of its ^{15}N to other amino acids during metabolism. Also it gives a plasma plateau enrichment in a reasonable time period and is readily separated by liquid chromatography. However, they were unable to measure the intracellular free lysine enrichment and used the plasma lysine enrichment in the calculation, thereby, underestimating the actual muscle protein fractional synthetic rate. Furthermore, the fractional synthetic rate of the sarcoplasmic fraction of the muscle was calculated from the total ^{15}N enrichment rather than that of [^{15}N]lysine. This would overestimate the fractional synthetic rate since the long infusion of [^{15}N]lysine would result in labelled glutamate and arginine being incorporated into the sarcoplasmic fraction. However, accepting these limitations it was reported with this method that total muscle protein synthesis accounted for 53% of whole body protein turnover, confirming the importance of muscle protein synthesis in man.

Stein et al. (1978a) used a continuous infusion of [^{15}N]glycine to measure protein synthesis in liver, tumour and normal gut of patients with gastrointestinal cancer. A criticism of the method used in this study is that protein synthesis rates were estimated from the ^{15}N incorporated into tissue protein and the ^{15}N enrichment of the tissue free amino acid pool rather than [^{15}N]glycine. The use of the ^{15}N enrichment to measure protein fractional synthetic rates will be less accurate than using the tissue free and bound [^{15}N]glycine enrichment, since the relative concentration of tissue bound amino acids is not the same as the relative concentration of tissue free amino acids (Munro, 1970). Furthermore, there are significant differences in the alpha-amino nitrogen enrichment of free amino acids during continuous infusion, even when using [^{15}N]glycine which readily transfers the amino nitrogen to other amino acids (Matthews et al.,

1981b). Indeed, using the two methods, a significant difference in the measured liver protein fractional synthetic rate has been reported, the [^{15}N]glycine derived synthesis rates were approximately double the ^{15}N derived values (Stein et al., 1980). Therefore, the accuracy of the fractional synthetic rate obtained by this method is in doubt (Stein et al., 1980). Nevertheless, this study was the first attempt in man to measure rates of fixed protein synthesis in the liver.

Reflecting the move away from [^{15}N]glycine to [^{13}C]leucine for whole body protein turnover measurements, the next tissue measurements in man were made in skeletal muscle using a primed continuous infusion of [^{13}C]leucine for 8h (Rennie et al., 1982a). The method that was used was similar to that of Halliday and McKeran, (1975) and whole body and muscle protein synthesis were measured simultaneously. Plasma alpha-ketoisocaproate enrichment was used to calculate the leucine flux and the muscle fractional synthetic rate. It was assumed that the plasma alpha ketoisocaproate enrichment was a better estimate of the intracellular precursor (leucine) enrichment. This method has been the basis of muscle protein synthesis measurements made since. It has been used to investigate the effect of feeding (Rennie et al., 1982a), muscular dystrophy (Rennie et al., 1982b), immobilisation of the leg (Gibson et al., 1987), and idiopathic scoliosis (Gibson et al., 1988) on muscle protein synthesis.

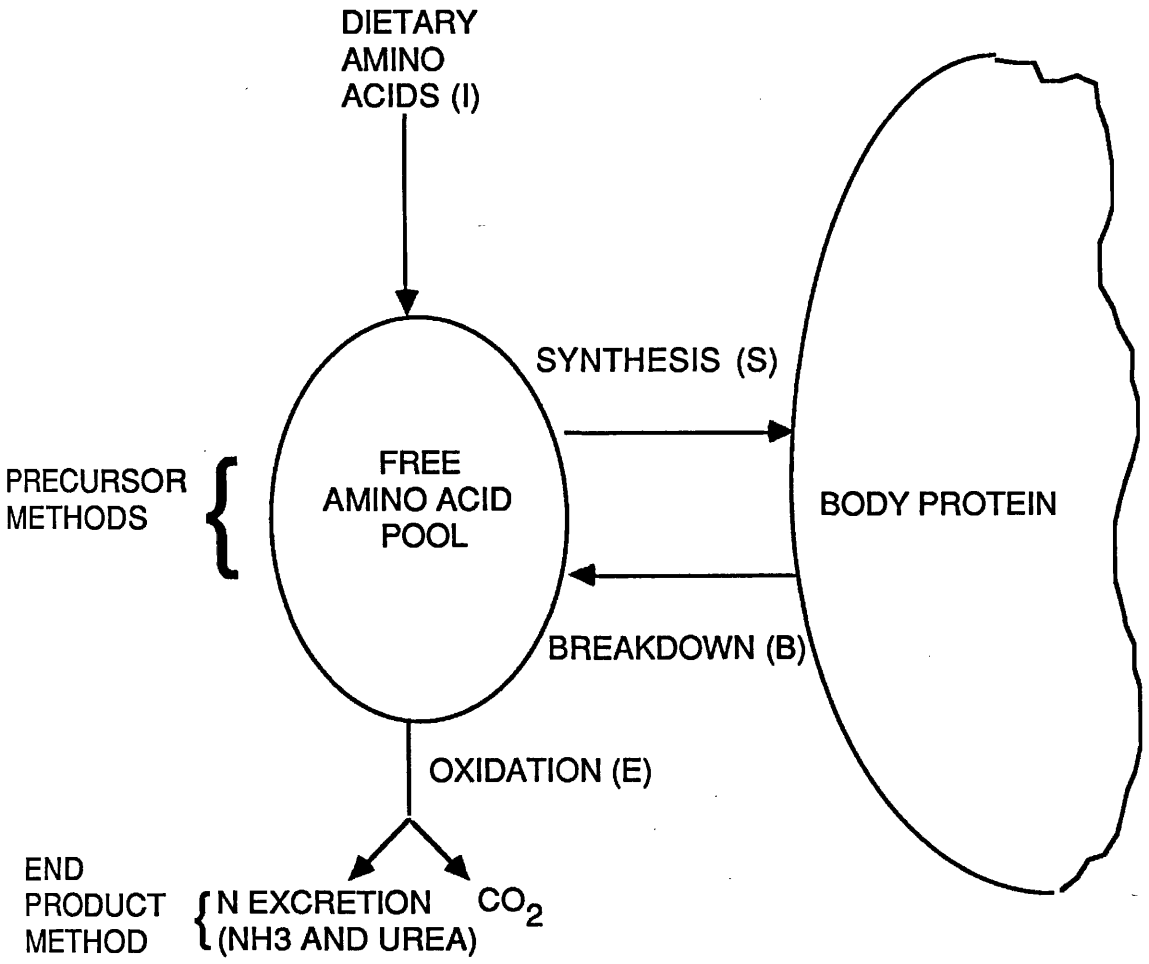
2.6 Flooding dose protocol

As already stated, calculation of the rate of protein synthesis requires not only measurement of the incorporation of the labelled amino acid into the protein but also the enrichment of the free amino acid at the site of protein synthesis. The latter has proven a difficult measurement to make using a continuous infusion of a tracer dose of labelled amino acid.

This difficulty is due to compartmentation of the free amino acid in the tissue and the problems associated with measuring the enrichment of the actual precursor for protein synthesis, that is aminoacyl-tRNA. Alternatively, the 'flooding dose' technique, in which the labelled amino acid is given with a large amount of the unlabelled amino acid as a bolus could be used. The basis of this method is that the high concentration of the free amino acid results in rapid equilibration of the amino acid in the intra- and extracellular compartments of the tissues, including the pool that is used for charging tRNA, to similar isotopic enrichments. Therefore, the plasma free amino acid enrichment can be used to estimate the fractional synthetic rate with little error. This method has been used to measure protein synthesis in cultured cells (Ballard, 1982; McNurlan and Clemens, 1985) perfused or incubated tissues in vitro (Mortimore, Woodside and Henry, 1972; Fulks, Li and Goldberg, 1975) and animal tissues in vivo (Henshaw et al., 1971; Scornik, 1974; McNurlan, Tomkins and Garlick, 1979; Garlick, McNurlan and Preedy, 1980). The advantage of accuracy together with rapid and convenient measurements of protein fractional synthetic rates has resulted in this method being validated recently for the measurement of muscle protein synthesis in man (Garlick et al., 1989). However, this technique, developed initially in animals to measure tissue protein synthesis, does not provide an index of protein synthesis in the whole body unless the tracer incorporation is measured in most tissues and the organ protein masses are known (Attaix et al., 1988). Moreover, the flooding dose protocol would fail to resolve fixed from export protein synthesis in the liver (since in the short time course of the flooding dose protocol tracer incorporation into a tissue biopsy would reflect both fixed and export components). Therefore, the flooding dose approach was not consistent with the aims of this thesis.

2.7 Summary

To satisfy the aims of the thesis (see section 1.6) it is necessary to measure simultaneously whole body, liver and skeletal muscle protein synthetic rates, to separate the contribution to total liver protein synthesis between fixed and export components and to make these measurements within a protocol such that the tissue biopsies are obtained at the start of an abdominal operation. From the review of the literature presented above, it is clear that rather than a flooding dose technique, the stochastic approach together with a tracer dose of labelled amino acid best allows these aims to be achieved. However, what is not clear is whether [^{13}C]leucine (precursor method) or [^{15}N]glycine (end-product method) might give the best estimate of tissue synthetic rates. Thus, in this thesis a comparison was undertaken in a group of healthy controls between [^{13}C]leucine and [^{15}N]glycine (Chapter 5) and the tracer which gave the most reliable results was used to study a group of weight-losing cancer patients (Chapter 6).



WHOLE BODY PROTEIN TURNOVER (Q) = S+E = B+I

In the fasting state: Breakdown = Q
Synthesis = Q - E

Figure 2.1 Two pool model of whole body protein metabolism

CHAPTER 3: METHODS: STABLE ISOTOPE SAMPLE PREPARATION, BIOCHEMICAL ANALYSES AND MEASUREMENT OF RESTING ENERGY EXPENDITURE

3.1 Introduction

A number of ^{13}C or ^{15}N-labelled amino acids have been used to make measurements of protein synthesis in man. With few exceptions analysis of precursors labelled with ^{13}C or ^{15}N has been carried out by Gas Chromatography-Mass Spectrometry usually in the selected ion monitoring mode. An attempt has been made to measure the ^{15}N enrichment of lysine by Isotope Ratio Mass Spectrometry (Halliday and McKeran, 1975), though the method was cumbersome and required 30ml of blood for each sample. Such a method was unacceptable for most clinical studies. Thus the Isotope Ratio Mass Spectrometry method of measuring precursor enrichment was at a considerable disadvantage compared to Gas Chromatography-Mass Spectrometry techniques capable of making the isotope analysis in less than 1ml of plasma. However, as discussed in Chapter 2, to obtain the maximum information (oxidation data) from turnover studies it is necessary to make an end-product measurement which requires Isotope Ratio Mass Spectrometry. Also, Gas Chromatography-Mass Spectrometry is not suitable for measuring ^{13}C or ^{15}N enrichment of labelled substrates incorporated into biopsy samples (which is usually of low enrichment). There are also methodological problems with this approach not least the requirement that both the Gas Chromatography-Mass Spectrometry and Isotope Ratio Mass Spectrometry instrumentation are calibrated with one another and remain so throughout the measurements. These disadvantages together with the fact that there are very few laboratories in a clinical situation equipped with both types of

mass spectrometer have resulted in few studies of this type being carried out. An aim of this thesis was to develop methods by which both precursor and end-product ^{13}C and ^{15}N measurements could be made on the same mass spectrometry system, i.e. Continuous Flow-Isotope Ratio Mass Spectrometry. The Continuous Flow-Isotope Ratio Mass Spectrometer that was used in these studies has been shown to be accurate, precise and have good precision for the isotope analysis of CO_2 and N_2 after quantitative oxidative combustion (Preston and Owens, 1983, 1985; Preston and McMillan, 1988). However, as the isotope analysis is carried out after combustion of the precursor or end-product sample it is essential that these compounds are pure since if they are not, isotopic enrichment measurements will be inaccurate. This is in contrast to sample preparation in radioisotope tracer studies since contamination of the sample by any source of nitrogen or carbon (eg. from organic or aqueous solvents) has to be removed prior to combustion and isotope analysis. Therefore, a key step of the sample preparation in this work was the amino acid separation of leucine and glycine from the complex sample matrices of plasma, intracellular fluid and hydrolysed protein.

3.2 Isolation of free amino acids from biological fluids prior to amino acid separation

The biological fluids in man which are most commonly analysed for free amino acids are blood, plasma (or serum), and intracellular fluid. The chief feature of these sample matrices is the presence of large amounts of protein, and therefore degradation of these proteins could potentially contribute a large amount of low enrichment amino acids to the free pool. Also, in order that the best separation of amino acids be obtained, it is important that the free amino acids as a group are removed from all

interfering compounds. Therefore, the free amino acids should be separated from potentially interfering compounds, rapidly, in controlled conditions.

3.2.1 Removal of protein and interfering compounds

The first step in preparation of a (plasma or serum) sample for free amino acid separation is to remove protein, including any enzymes which might bring about their breakdown. The simplest and longest established method is to precipitate all protein and isolate the supernatant, the protein being denatured by the precipitant. Popular acidic reagents for protein precipitation are trichloroacetic acid, perchloric acid, sulphosalcylic acid and tungstic acid. These methods using acid precipitation leave large molecular weight lipids in solution. To avoid interference from these methanol and ethanol have been used, at least two volumes of ethanol being required to precipitate all plasma proteins (Dell, 1967). Both acid and alcohol methods of protein precipitation, because their action is through denaturing proteins, can give variable yields of free amino acids and compromise recovery of proteins from the sample. To avoid these problems ultrafiltration methods (using commercially available filters) have become increasingly popular.

In the work presented in this thesis ultrafiltration was used to remove protein (and all compounds of molecular weight greater than 25,000 daltons) from plasma and homogenate samples (Figure 3.1, 3.2). The suitability of ultrafiltration was verified by analysis of the amino acid content before and after ultrafiltration of a plasma sample (Table 3.2). It would appear from the data obtained that the ultrafiltration did not selectively retain any of the amino acids measured and that the recovery of amino acids was on average 83%. These measurements were in accord

with the recovery of [^{14}C]glycine (Sigma Ltd, Poole, UK.) added to plasma samples (Table 3.1). Further purification of the amino acids was achieved by batch cation exchange resin (Figure 3.1, 3.2). Recovery of [^{14}C]glycine following this step was determined to be approximately 90% (Table 3.1).

3.2.2 Protein hydrolysis

There are three principal methods for cleaving peptide bonds to prepare hydrolysates suitable for amino acid separation. Each has advantages and disadvantages.

(a) Acid Hydrolysis

Several acids have been used to hydrolyse peptide bonds. Each has some advantages with reference to individual amino acids, and none are without some undesirable features, such as the introduction of artifacts or the partial or complete destruction of some amino acids (Hill, 1965). The most commonly used method employs 6N HCl at 110^{o}C for 24h or longer. However, higher temperatures (up to 155^{o}C) have been employed with concomitant shortening of the time required to complete the hydrolysis. Although the destruction of some amino acids is also increased, proper correction can be applied. The protein or peptide in dry form is placed in a suitable vessel, the acid added, and the oxygen present removed by a nitrogen flush, evacuation or a combination of both. The removal of oxygen is an important step in the procedure and is often difficult because solutions of peptide or denatured protein tend to foam under vacuum. This can be reduced by freezing and thawing slowly under vacuum. Vortexing during the evacuation stage is equally effective and much faster. After hydrolysing the protein, the HCl is removed by rotary evaporation or vacuum distillation. This is preferable to drying overnight in a dessicator in

terms of both efficiency and the prevention of artifacts formed under anhydrous conditions (Ikawa and Snell, 1961) Dry samples can be reconstituted in water or an appropriate buffer for subsequent separation.

(b) Alkaline hydrolysis

The greater range of amino acids destroyed in alkali has prevented this method from being widely adopted. Serine, threonine, arginine, and cystine are extensively or completely destroyed, and considerable racemisation, most notably of isoleucine (to form allo-isoleucine, which is resolved from the natural isomer of isoleucine on automatic analysers), is observed. The most common application of this method is for the determination of tryptophan.

(c) Enzymatic hydrolysis

There are several advantages in using enzymes to hydrolyse peptide bonds. Firstly, the enzymes are specific and readily cleave bonds that are resistant to non-specific catalysts. Second, they are non-destructive, not only to the common amino acids, but also to labile groups introduced either in vivo or in vitro. Finally, they act rapidly and in theory could complete the hydrolysis in a matter of only a few minutes. In practice, these advantages are largely offset by the fact that the enzyme-catalysed hydrolysis of peptide bonds does not proceed materially faster than other catalysts and, more importantly, often does not go to completion. The latter observation reduces the general utility of this method and confines its use primarily to cases in which identification of labile amino derivatives is the principal point of interest.

In order to obtain maximum recovery of the neutral amino acids, glycine and leucine, conventional acid hydrolysis was used in this thesis. However, instead of the protein being hydrolysed in 6M HCl at 110°C for 24h it was carried out at 145°C for 4h (Roach and Gehrke, 1970). This was done to increase the throughput of samples in line with other parts of the sample analysis.

3.3 Amino acid separation

3.3.1 Introduction

The early stages of amino acid analyses date from the discovery of glycine by Henri Braconnot in 1820. This was the first time in which a pure amino acid was obtained from an acid hydrolysate of a protein, although asparagine and cysteine had been observed a decade earlier. The succeeding century saw the identification of most of the remaining common amino acids, although threonine was not discovered until 1935. The isolation of the amino acids and their subsequent determination in other samples were accomplished using macroscopic methods that require large amounts of protein as starting material. As analytical procedures they suffered from inaccuracy due to the large losses encountered and from the extensive amount of time and effort required. Despite the overall limitations of the procedures in producing quantitative analyses, certain amino acids that contain appropriate functional groups were readily determined at considerably more sensitive levels by the use of colorimetric methods. Folin and Denis (1912) introduced such an approach to the measurement of tyrosine and tryptophan, and procedures for the determination of arginine (1925), histidine (1919), and phenylalanine (1932) were reported subsequently. The highly reactive thiol group of cysteine has also been

utilised for analysis of this amino acid in a number of different systems. Other approaches to amino acid determination included solubility product procedures (Moore, Stein and Bergmann, 1942), the isotope dilution method (Rittenberg and Foster, 1940), the isotope derivative method (Keston and Udenfriend, 1949), and periodate oxidation for the determination of serine and threonine (Shinn and Nicolet, 1941; Rees, 1946). In addition, procedures employing the specificity of enzymes and the nutritional requirements of microorganisms were developed, primarily in the 1940's that allowed the complete determination of a mixture of amino acids without prior fractionation.

The introduction of chromatographic techniques, which permitted the complete separation of all the amino acids prior to their estimation and which form the basis of present methods of analysis, began with the studies of Martin and Synge (1941), who used silica gel columns for separating N-acetyl amino acids. The use of paper chromatography to separate free amino acids followed directly afterward (Consden, Gordon and Martin, 1944). Partition chromatography of amino acids on starch columns was reported by Elsden and Synge (1948) and Stein and Moore (1948) and was further developed by the latter workers (Moore and Stein, 1949; Stein and Moore, 1949a). This line of experimentation led, in turn, to the introduction of ion exchange resins as the separation medium of choice (Moore and Stein, 1951) as well as the invention of the fraction collector and the use of ninhydrin as a universal reagent for detecting and quantifying amino acids both manually and automatically (Moore and Stein, 1948).

Although the introduction of ion-exchange resins, particularly sulfonated polystyrene, led directly to automated quantitative analyses, paper chromatography enjoyed widespread use for a number of years as

both a qualitative and quantitative technique. Paper chromatography is no longer widely used for amino acid analysis as it has poor sensitivity compared to modern automated ion-exchange instruments.

3.3.2 Ion-exchange chromatography

The separation of amino acids by ion-exchange is primarily due to differences in pKa values and follows the general order: acidic, neutral and finally basic amino acids. Various interactions between structural features (e.g. aromatic rings or non-polar side chains) of amino acids, and the resin modify retention properties. The cation-exchange resin is usually made by sulphonation of beads of styrene, and copolymerised with di-vinyl benzene.

The complete resolution of the normal protein-bound amino acids was first achieved on columns of cation exchange resin (sulphonated polystyrene, Dowex 50x8) in 1951. Operated in the sodium form, the column development was accomplished with multiple buffers covering the range pH 3.4-11.0 at temperatures from 25°C to 75°C (Moore and Stein, 1951). Two columns were required, one for the acidic and neutral amino acids and one for the basic ones. Since that time, improvements in almost every phase of this type of analysis have been made. Continuous refinement, particularly with respect to the physical state of the resin (Benson, 1972), has allowed a steady reduction in the size of the columns required. These changes have led directly to the practicability of the separation of all the amino acids using one column, from increasingly smaller loaded samples.

The constant molarity single column system described by Hare (1972) utilises stepwise buffer solutions of increasing pH and a constant sodium concentration of 0.2mol/l. The latter method is advantageous because the minimal changes in ionic strength of the various eluents

provide an environment in which resin swelling and shrinkage are minimised. Thus, low pump pressures can effect relatively high eluent linear flow velocities and thereby allow a reduction in total analysis time.

Constant molarity eluents were utilised, not only to reduce pump pressures, but also because anions are available that can be used to prepare solutions with high buffering capacities over a wide pH range. The latter consideration was extremely important in providing sufficient flexibility to develop eluents capable of separating the various amino acid derivatives.

Similar developments leading to improved elution buffers and detecting reagents have been important. Along with the improvements in the chemistry of automated ion-exchange analysis of amino acids, there has been considerable advancements in instrumentation. These include improvements in eluent delivery, monitoring devices, and computational aids.

In modern amino acid analysis by cation exchange chromatography amino acids are displaced from the column in discrete bands by varying buffer pH or ionic strength. The buffer systems that are used are changed with the sample type, protein hydrolysate or physiological fluid, and are determined by the counterion used (usually sodium or lithium) and by the method of buffer changes applied to the resin (stepwise or gradient elution). The buffering component commonly used is citrate which is suitable for solutions below pH 7 (Bates and Pinching, 1949). Citrate concentrations of 0.05 to 0.067mol/l are common. Buffers are prepared either with citric acid or an alkali salt. Unfortunately, for high sensitivity work, the largest reagent contribution to amino acid contamination is citric acid. If consistent analyses are to be achieved it is essential that high purity reagents are used for buffer preparation. Finally, there is no

theoretical limit to the lifetime of the ion exchange column, as there is with silica columns. Therefore, if care is taken in use of eluants and if samples are carefully prepared, to avoid injecting particulates onto the column, the columns can separate thousands of biological samples.

3.3.3 Separation of derivatised amino acids

The many variations of the partition, paper, and ion-exchange chromatographic procedures that have been used for the determination of free amino acids have also been employed in conjunction with derivatisation of the amino acids. Notable among these are dinitrophenylation, introduced by Sanger (1945) and developed for this purpose by Levy (1954), and the Edman (1950) reaction as developed by Sjoquist (1955). Derivatisation to provide volatile products is also required for gas-liquid chromatographic analysis of amino acids. Although initially promising because of its speed and sensitivity (Zumwalt, Roach and Gehrke, 1970), it has not yet proved to be sufficiently reliable to replace ion exchange methods for routine amino acid analysis in biological fluids.

High performance liquid chromatography has undergone rapid development in amino acid separation (Molnar and Horvath, 1977; Kraak, Jonker and Huber, 1977; Hancock, Bishop and Hearn, 1979). Alternatives to ion exchange chromatography have appeared in the last few years to allow simple and straightforward occasional analysis of amino acids. These are pre-column derivatisation methods using reverse phase High Performance Liquid Chromatography which are rapid and sensitive. Two of the most widely used methods involve the formation of dansyl (Wiedmeier, Porterfield and Hendrich, 1982; Dejong et al., 1982) and o-phthalaldehyde (Lindroth and Mopper, 1979) derivatives of the amino acids prior to High Performance Liquid Chromatography analysis. Both methods give greater

sensitivity and shorter analysis time when compared to post column derivatisation techniques. At present, the reverse phase High Performance Liquid Chromatography method although suitable for some applications is not routinely used because of some difficulty in determining certain amino acids. A method has recently been reported that describes quantitative derivatisation of amino acids with phenylisothiocyanate, followed by the separation and quantitation of the resulting phenylthiocarbamyl derivatives by reverse phase High Performance Liquid Chromatography (Heindrikson and Meredith , 1984). These derivatives are stable enough to eliminate any need for in-line derivatisation. However, the use of reverse phase high performance liquid chromatography for amino acid separation with need for derivatisation complicates the enrichment measurement by Isotope Ratio Mass Spectrometry (see below, Chapter 4).

3.3.4 Separation of amino acids using ion-exchange with a volatile buffer: Development work

Introduction; Accurate isotopic enrichment measurement of amino acids by Isotope Ratio Mass Spectrometry (see Chapter 4.) require that the amino acid be pure since contaminants are most likely to alter the measured enrichment of ^{13}C or ^{15}N. In order to obtain pure amino acids from biological samples for isotope analysis the use of cation exchange chromatography in conjunction with a volatile eluant was investigated. Although analysis of amino acids by ion-exchange chromatography has been repeatedly refined since its development in the 1950's, far less work has been directed towards refining separation and isolation of pure amino acid by ion-exchange chromatography. This has been mainly because the only practical use for such a system has been the isolation of isotopically labelled amino acids from biological sources. From the literature, it would

appear that two volatile eluants have been used, one using HCl (Hirs, Moore and Stein, 1954), the other based on pyridine formate (Liebster, Kopoldova and Dobiasova, 1961). The use of HCl as an eluant to obtain pure leucine and glycine was more complex than that described for pyridine formate, involving not only changing the concentration of the eluant but also the temperature during the amino acid separation. Furthermore, the fractions eluted from the cation exchange column contain brown residues from the ion exchange resin which may not be completely removed by a further anion exchange step (Hirs, Moore and Stein, 1954). The pyridine formate eluant, in contrast, leaves no residue after lyophilisation and has the advantage of separating amino acids at a fixed temperature (37°C). Also the pyridine formate buffer system gives a stable pH (reducing variability in the amino acid separation) and is a less aggressive eluant (to the stainless steel equipment) for high performance liquid chromatography. Furthermore, from the report of Liebster and coworkers (1961) it appeared that the separation of leucine and glycine could be carried out using a single buffer although glycine appeared to coelute with alanine. The column used in the separation described by Liebster and coworkers (1961) was 2.0x150cm filled with Dowex 50x4 (200-400mesh), operated at 37°C, and loaded with 600mg of amino acids. This work was confirmed by Cohen and Putter (1970) who scaled up the amount of amino acids separated by approximately 10 fold. The column they used was 7.5ftx2.0in and the resin AG50x8 (400 mesh, Biorad, CA, U.S.A.) and operated at 37°C. However, the resolution of the amino acid separation became poorer (Cohen and Putter, 1970; Cohen, Horsely and Sternlicht, 1970).

Therefore, in order to resolve glycine and leucine from the other amino acids a high resolution cation exchange column (4.6 x 250mm,

packed with a sulphonated polystyrene-divinyl-benzene copolymer, stable with solutions of pH 0-14, Amino Acid Analysis column AA911, Interaction Chemicals Inc., California, USA.), in which the spherical particle size was almost uniform (9.0$\pm$0.5um), was used. The theoretical column loading of amino acids is approximately 16mg (100mg/cm^2 cross sectional area, Instruction Manual, Interaction Chemicals Inc., CA, U.S.A.). This column was used in conjunction with an isocratic high performance liquid chromatography system (see section 3.4.3). Furthermore, since resolution is improved by ensuring that non-amino acid components, especially cationic species, are removed from the biological sample, an extensive amino acid clean-up procedure was carried out (see section 3.4.1 and 3.4.2).

Methods; Pyridine formate buffer was made by adding formic acid (BDH, U.K.) to a given molarity (0.2mol/l) of pyridine (BDH, U.K.) in 980ml of water until the correct pH of the solution was reached, measured by a calibrated pH meter (PHM62, Radiometer, Denmark). This solution was made up at the time of amino acid separation and used for a maximum of 4 days from this time. The pH of the pyridine formate solution was checked daily and stored in a dark brown glass bottle. The protein-free sample is dissolved in pyridine formate, pH2.1, for application onto the column of cation-exchange resin. When the sample pH is 2.1 ionisation of the amino acid carboxyl groups is suppressed and they are retained on the column of cation exchange resin via their charged amino groups.

The standard solution used in the examination of the amino acid separation was a mixture of neutral amino acids (L-alanine, glycine, L-valine, L-isoleucine, L-leucine, Sigma Chemical Co. Ltd., U.K.) at the concentration of 20umol/ml in 0.2mol/l pyridine formate pH 2.1.

The retention time and purity of leucine and glycine were assessed as follows; The retention time of the injected amino acids was measured by a spot test on each fraction collected by a fraction collector (0.4min/160ul) of the amino acid separation. 2cm squares were ruled and numbered in pencil on Whatman No.1 filter paper (BDH Chemicals Ltd, U.K.). Aliquots (approximately 4ul) were then spotted with capillary micropipettes, dried and sprayed with a 0.3% solution of ninhydrin in acetone (BDH Chemicals Ltd, U.K.) and the colour was allowed to develop for 20min at 90°C.

The purity of leucine and glycine in the eluant fractions was assessed by thin layer chromatography using a butanol/acetic acid solvent system (Smith, 1969). 1ul of the fraction or standard solution (see above) was spotted on to a thin layer chromatography plate (silica gel 60 F_{254} plates, BDH Chemicals Ltd, U.K.) 1cm apart and 2cm from the bottom of the plate. The plate was then run for 5h using a butanol/ acetic acid/ water solvent system (60/15/25, BDH Chemicals Ltd, U.K.) in a glass tank. The plate was then dried overnight and the amino acids visualised with ninhydrin as described above.

Using the equipement and methods described above, the conditions of eluant flow, molarity and temperature were considered to optimise the amino acid separation with particular reference to leucine and glycine.

Eluant Flow; The maximum operating pressure of the column used (AA911, Interaction Chemicals Inc., CA. U.S.A., see 3.4.3) is approximately 2500 pounds per square inch (higher pressure would result in permanent distortion of the spherical particles). The recommended flow rate for the cation-exchange column was 0.5ml/min using a conventional citrate buffer system, which according to the manufacturers, would generate an eluant pump pressure of approximately 1700pounds per

square inch. However, when 0.2mol/l pyridine formate was pumped through the column this pressure was reached at 0.4ml/min flow rate. Therefore, with regard to the safe operating pressures and since a small improvement in resolution accompanies reductions in flow rate (AA911, amino acid analysis column instruction manual, Interaction Chemicals Inc, CA, USA.) 0.4ml/min eluant flow rate was used.

Molarity; Liebster and coworkers (1961) used 5 different buffers to carry out the separation of all the amino acids from protein hydrolysates. This essentially involved using different concentrations of pyridine (0.1-2.0mol/l) adjusted to a different pH (3.1-5.1) with either formic acid or acetic acid. In this work the separation of only two amino acids were of interest, leucine and glycine. These amino acids occur in the same region of the amino acid separation with the pyridine formate eluant and this raised the possibilty that the separation of leucine and glycine can be simplified. One buffer (pyridine formate pH 3.1) at different concentrations (0.1, 0.15, 0.20, 0.25mol/l) was used to determine whether both leucine and glycine could be separated with a single buffer and how long this would take. 50ul of the amino acid standard solution at pH2.1 was injected (see above) onto the high performance liquid chromatography column (see 3.4.3) and fractions collected (0.4min/160ul) in the region of the neutral amino acids.

The effect of the concentration of pyridine formate on the retention time and purity of the amino acids was examined (Table 3.3). There was a reduction of approximately 10min in retention time of the amino acids in the standard mixture for each 0.05mol/l increase in pyridine formate concentration. This resulted in the chromatogram being compressed into fewer fractions until at 0.25mol/l pyridine formate there were no clear fractions between alanine/glycine and isoleucine/leucine (as assessed by

the spot test). Furthermore, alanine/glycine and isoleucine/leucine were found in the same fractions. Therefore, 0.25mol/l pyridine formate was not used. There was complete separation of the amino acids in the standard mixture using 0.1, 0.15 and 0.2mol/l pyridine formate at 50°C assessed by the procedures described above. However, using 0.2mol/l pyridine formate with collection of fractions every 0.5min (200ul) the whole glycine or leucine peak was in less than 10 fractions. Therefore, the concentration of pyridine formate used was 0.2mol/l.

Temperature; The effect of temperature of the amino acid separation on the retention time and purity of the amino acids was examined (Table 3.3). There was approximately a 10% reduction in retention time of the amino acids in the standard mixture, using 0.2mol/l pyridine formate, when the temperature of the elution was increased from 40°C to 50°C and 50°C to 60°C. However, the separation carried out at 60°C or above was less reliable due to bubble formation in high performance liquid chromatography system. It appeared that the bubble formation was originating at the exit of the column. Therefore, the operating temperature of the column was chosen to be 50°C.

In order to assess the amino acid separation of glycine and leucine in a more complex amino acid mixture L-alanine-UL-^{14}C (UL, uniformly labelled, glycine-UL-^{14}C, L-isoleucine-UL-^{14}C and L-leucine-UL-^{14}C were added to plasma samples and treated as described in section 3.4.1 and 3.4.3. The separation of the labelled amino acids was carried out on a different days with freshly prepared 0.2mol/l pyridine formate at 50°C and the eluted fractions were counted (Table 3.4). The fractions were collected at 2.5min intervals to check the retention time of the amino acids, subsequently fractions were collected at 0.5min intervals over this period (20-55min). These results confirm the separation of glycine and leucine

from alanine and isoleucine respectively and that the separation was consistent with different batches of buffer. Finally, using 0.2mol/l pyridine formate and a flow rate of 0.4ml/min, the separation of all the amino acids (acidic and neutral) was defined in a tissue hydrolysate. A liver biopsy sample was treated as described in sections 3.4.2 and 3.4.3 and the eluted fractions underwent amino acid analysis (Rank Hilger Chromaspek M aminoacid analyser). This demonstrated that the amino acid pairs of glycine/alanine and leucine/isoleucine were resolved from one another (Figure 3.3).

Detection Direct spectrophotometric detection of free amino acids, after cation exchange chromatography using conventional citrate buffers is not generally useful since many other organic molecules absorb at similar wavelengths (e.g. 190nm). However, pyridine formate has a large U.V. absorption and this raised the possibility that underivatised amino acids such as valine, glycine, alanine, leucine and isoleucine may be visualised as negative peaks. In order to confirm whether this was the case the optimum wavelength for the continuous monitoring of the amino acid separation was determined to be 278nm by scanning (190-800nm) 0.2mol/l pyridine formate (DU 7 spectrophotometer, Beckman Instruments, High Wycombe, UK.) before and after the addition of 1umol leucine and glycine (Sigma Chemical Co. Ltd, U.K.). Therefore, an ultraviolet detector (deuterium arc lamp, diffraction grating monochromator bandwith of 8nm, two vacuum photocells and 8ul flow cell, Pye Unicam, PU4020UV detector, Cambridge, UK.) at 278nm, connected to a chart recorder (Servoscribe 1S, Smiths Industries Ltd, U.K.) was used to monitor the amino acid separation. On elution of each of these amino acids from the column there was indeed a negative peak due to the displacement of the U.V. absorbing pyridine (Dolan, 1988). This was confirmed by using ^{14}C

labelled leucine and glycine (see Table 3.4). Therefore, this method of detection, although not developed into a quantitative technique, does give a recognisable elution pattern and enables the separation of leucine and glycine to be monitored. A typical trace from the detector, at different sensitivities, is shown in Figure 3.4. The trace is the same whether the injected amino acids are from plasma, intracellular fluid or tissue hydrolysate.

3.3.5 Summary

It is more than 30 years since the separation of amino acids by ion exchange chromatography was first reported. Although gas-liquid chromatography and reverse phase high performance liquid chromatography have attempted to rival this system, it still remains the most popular and probably the most reliable method of separating amino acids. With the introduction of polymeric ion-exchange columns being used in a High Performance Liquid Chromatography system the separation of all the common amino acids can be carried out in under 1h (Benson and Woo, 1984). This approach has been used in this thesis for several reasons. Firstly, it enables fast, reproducible separation of amino acids. Secondly, the amino acids do not have to be derivatised except for post-column detection, which avoids dilution of the tracer label. Thirdly, the ion-exchange separation of amino acids can be carried out with volatile buffers enabling the chemical contribution of the solvent to the amino acid fraction to be kept to a minimum.

3.4 Sample preparation for isotope analysis

All reagents used were analytical grade and water was doubly distilled, deionised (18Mohm). In the recovery experiments carried out with radioactive tracers, [^{14}C]leucine and [^{14}C]glycine (Sigma Chemical Co. Ltd, Poole, UK.) specific activity measurements were made using a Sample Channels-Ratio method (scintillant, Optifluor; scintillation counter, Tri-Carb; Packard Instrument Co. Ltd., Illinois, USA.) Each vial was counted until 10,000 radioactivity counts had accumulated. The Channels-Ratio method was used since it has the advantage of giving simultaneously both the sample count rate and the channels-ratio. The sample is counted once and no additional manipulation is required. The disadvantage of the method is that large errors are incurred in samples which are highly quenched or have low count rates (Rogers and Moran, 1966). However, the samples that were analysed in this work were usually of a high count rate (>1000 DPM) with similar composition and were not highly quenched.

3.4.1 Plasma Sample Preparation (Figure 3.1)

5ml of plasma was deproteinised by centrifugation at 1000G for 35min through an ultrafiltration cone, molecular weight cutoff <25000daltons (Amicon Ltd, UK.). The retained material was then washed with 5ml water. This was repeated with 5ml 0.15mol/l potassium chloride (KCl). The deproteinised plasma was then acidified with 1.5ml 0.1mol/l hydrochloric acid (HCl) prior to purification using 4ml cation exchange resin (H+ form AG50W-X8, 200-400 mesh Biorad laboratories, UK.). This step, carried out also in the tissue sample preparation, introduces a further molecular weight separation since all compounds of a molecular weight greater than 1000daltons are excluded from binding on to the resin and washed off. The purified amino acids were eluted with 5 bed volumes

7mol/l ammonium hydroxide (NH_4OH) collected in a pear shaped flask and freeze-dried. The freeze-dried mixed free amino acids produced from the above sample preparation is simple and close as possible to a standard amino mixture to facilitate separation.

3.4.2 Tissue Sample Preparation (Figure 3.2)

The methods used in the preparation of tissue samples were directed at obtaining both the intracellular free glycine and free leucine enrichment and also that of incorporated amino acids in the same sample without significantly altering the composition of the sample (i.e. without degrading the sample, adding cations or altering pH). The whole procedure from weighing tissue to the preparation of soluble and non-soluble material was carried out at 4°C in a cold room, in order to minimise tissue breakdown (e.g. due to enzymic hydrolysis) prior to protein separation. The liver tissue was weighed and then homogenised with 4ml water at 4°C. Muscle tissue was ground with dry ice by mortar and pestle prior to homogenisation. Water homogenates have been used successfully for systems in which enzymic activity has been measured provided that the tissues are not left at room temperature for more than 15min (Potter, 1955). Following centrifugation a sample of the homogenate supernatant (representative of the intracellular fluid, 2x100ul) was taken for amino acid analysis (see section 3.5.3) and frozen at 30°C until analysis. This treatment allowed the protein precipitant best suited for the amino acid analysis system to be used (sulphosalicylic acid, Graser et al., 1985, see section 3.5.3). 1ml of 1mol/l HCl was added and the liver and muscle re-homogenised. The separation of soluble and non-soluble material was carried out using HCl since it is a volatile acid and is compatible with the other sample preparation steps. This was then centrifuged at 1000G for 35min to yield

soluble and non-soluble material. The soluble material, containing tissue free amino acids, was ultrafiltered and the free amino acids purified further by cation exchange and freeze-dried. The non-soluble material, containing tissue protein (intracellular and extracellular protein), from which the incorporated glycine and other amino acids were extracted was washed 5 times with 10ml 0.5mol/l HCl to remove any trace of free amino acids. Preliminary [^{15}N]glycine enrichment measurements on the hydrolysed non-soluble protein from "normal" muscle samples gave enrichment values significantly higher (thus apparently high protein fractional synthetic rates) than would be expected from previous work. The origin of this increased [^{15}N]glycine enrichment in the muscle hydrolysate was considered to come from two sources. Firstly, the trapping of free [^{15}N]glycine in the non-soluble fraction and secondly contamination of the non-soluble fraction by an enriched protein/proteins. With reference to the first possibility the complete removal of labelled free glycine following this procedure was verified by mixing the non-soluble material with unenriched glycine followed by isotope analysis. Considering the second possibility, the protein component of the non-soluble material (solids) is composed of protein from intracellular solids and connective tissue solids. Furthermore, there will also be a protein contribution from the blood in the tissue (principally albumin) which, although thought to be less than 5% in normal muscle tissue (Rothschild et al., 1955), is potentially a source of protein which is at a higher [^{15}N]glycine enrichment than the muscle protein. However, the muscle biopsies in the present studies, although blotted, to remove excess blood, were not dissected free from blood and connective tissue as has been carried out by other workers (Rothschild et al., 1955; Garlick et al., 1989; Forsberg et al., 1991) and therefore the contribution of blood protein may have been greater than that estimated above. In order

to reduce the albumin contribution from blood, the muscle tissue was extracted by an acid/alcohol wash (Korner and Debro, 1956). This treatment reduced the [^{15}N]glycine enrichment in the hydrolysed non-soluble muscle fraction. It was confirmed by electrophoresis and immunofixation that albumin but not fibrinogen was extracted from the non-soluble muscle fraction and by electrophoresis that little other protein was extracted (see section 3.5.3; Figure 3.6). The remaining non-soluble protein was freeze-dried and 5mg was hydrolysed in 5ml 6mol/l HCl at 145°C under vacuum for 4h (Roach and Gehrke, 1970). The HCl was removed by vacuum distillation. This was followed by an ion exchange purification step and the sample freeze-dried (see section 3.4.1).

3.4.3 Amino Acid Separation

The resultant freeze dried material from plasma, intracellular and hydrolysate sample preparation was dissolved in 75ul 0.2mol/l pyridine formate pH 2.1. Using an isocratic high performance liquid chromatography system (Gilson Medical Electronics, Villiers le Bel, France), a 50ul aliquot of this solution was injected via a loop injector (7125, Rheodyne, CA, USA.) onto a 4.6 x 250mm cation exchange column (Amino Acid Analysis column AA911, Interaction Chemicals Inc., California, USA.). The amount of amino acids injected onto the column was well below the theoretical column loading of approximately 16mg (100mg/cm^2 cross sectional area, Instruction Manual, Interaction Chemicals Inc., CA, U.S.A.). The cation exchange column was maintained at 50°C with a column heater (Jones Chromatography, U.K.). The amino acids were eluted with 0.2mol/l pyridine formate pH 3.1. The flow rate was 0.4ml/min and the fractions were collected by time (0.5min/200ul) using an automatic fraction collector (7000 Ultrorac fraction collector, LKB Bromma, Sweden). This buffer

system gave optimum separation of the neutral amino acids although only glycine and leucine fractions were collected (see section 3.3.4). This approach yields pure amino acids when eluted fractions are freeze-dried and on combustion there is no detectable nitrogen residue from the pyridine formate buffer (see sections 4.2.2, 4.2.3). In addition, retention times, recovery and peak shape of glycine and leucine were checked using ^{14}C labelled glycine and leucine. Sample recovery was approximately 70% for the whole procedure from sample preparation to amino acid separation (Table 3.1), with approximately 50% of the original sample being used for isotope analysis. This procedure was not associated with significant fractionation of the isotope label (see section 4.2.5). Following the separation of the neutral amino acids, the remaining basic amino acids and ammonium were washed from the column with 0.2mol/l NaOH for 10min. Following the NaOH wash the column was re-equilibrated with 0.2mol/l pyridine formate. On the resumption of a steady baseline trace on the chart recorder (usually 50-60min after the NaOH wash) the next sample was injected. The time taken to separate glycine and leucine from the other amino acids was 32 and 52min respectively. The glycine and leucine peaks were collected in 10 x 200ul fractions (Figure 4.5, 4.4). These fractions were pipetted into pre-frozen aluminium combustion containers (12x5mm, Elemental Microanalysis, Okehampton, UK.) held in a drilled aluminium block at approximately -70 ^{o}C with solid CO_2 and freeze-dried for isotope analysis.

3.4.4 Urine sample preparation

Urinary ammonium was extracted using a sodium/potassium form cation exchange resin (AG50W-X8, 200-400 mesh Biorad laboratories, UK.) after Read, Harrison and Halliday (1982). 10ml of the urine sample

was added to 4ml of resin suspension (about 1g dry resin) in a universal container (Bibbey Sterlin Ltd, Staffordshire, UK.) and placed on a roller mixer (Luckham Ltd, Sussex, U.K.), at room temperature, for a minimum of 15min. The resin was then allowed to settle and the fluid above the resin was poured off. The resin, held in a universal bottle, was then washed with 10 ml water and the water discarded. In a modification of the method of Read, Harrison and Halliday (1982), 2ml 2.5mol/l potassium hydrogen sulphate was added to the resin and mixed as above to elute the bound ammonium. The resin was then allowed to settle and 50-100ul subsamples of the solution above the resin (25-100ug atom NH_4-N) were pipetted into pre-frozen combustion containers and freeze-dried for analysis.

After the removal of urinary ammonium using the batch cation exchange method described above, urea from an aliquot of the ammonium-free urine was converted to ammonium by hydrolysis with urease, and extracted using a second batch of the same cation exchange resin. Urea derived ammonium was then brought into solution from the washed resin by treatment with potassium hydrogen sulphate and 50ul aliquots (25-100ug atom NH_4-N) were freeze-dried into aluminium combustion containers (Preston and McMillan, 1988). Separation of urinary ammonium with this batch resin technique is quantitative and does not appear to fractionate ^{15}N in comparison with either aeration (Read, Harrison and Halliday, 1982) or microdiffusion (Preston and McMillan, 1990).

3.4.5 Breath CO2 sample preparation

Breath was sampled directly from a ventilated hood calorimeter (Kinney et al., 1964) using a 20ml disposable syringe fitted with a luer stopcock. It was verified that samples collected in this way can be stored

for up to 2 weeks, but were analysed within 24h. Three replicate samples for CO_2 isotopic analysis were taken at each collection point (Figure 5.1). The sample preparation for isotopic analysis of breath CO_2 by Continuous Flow-Isotope Ratio Mass Spectrometry (Preston and McMillan, 1988) is simpler and less expensive than the conventional approach which relies on cryogenic purification (Schoeller and Klein, 1978). The on-line purification of breath CO_2 is described in section 4.2.

3.5 Biochemical Analyses

Most of the analyses were carried out using in-house assays in Glasgow Royal Infirmary. The precision and accuracy of these assays were monitored by internal and external quality control schemes. Where precision values are given (coefficient of variation) the between batch variation represents the poorest precision with which the assay is carried out. The precision values obtained for these assays are comparable to that obtained by other laboratories participating in quality control schemes.

3.5.1 Urine analyses

Total urinary nitrogen; This was measured by a standard microkjeldahl method (Fleck and Munro, 1965).

Creatinine; Urinary creatinine was analysed on a Hitachi 704 discrete analyser based on the standard end-point Jaffe reaction.

Hydroxyproline; This assay was carried out using a standard method (Hodgkinson and Thompson, 1982) and the between batch variation was approximately 10%.

3-methylhistidine; concentrations were analysed on a Chromaspek M amino acid analyser and is based on a cation exchange resin, two buffer gradient elution, ninhydrin detection.

3.5.2 Blood analyses

Albumin; Plasma albumin was analysed by immunoturbidometric methods on an Encore^{R} centrifugal analyser (Baker Instruments). Antisera for the albumin was obtained from the Scottish Antibody Production Unit (Carluke, Scotland). The between batch coefficients of variation across the working range of the assay was <8%.

Fibrinogen; Plasma fibrinogen (EDTA) was estimated nephelometrically by measuring the light scattering intensity in diluted plasma by heat aggregation, with strict control of pH and temperature (Desvignes and Bonnet, 1981). The between batch coefficient of variation was <5% across the working range of the assay.

Fibrin degradative products; Crosslinked fibrin degradative products in plasma, containing the D-dimer, were measured by an enzyme immunoassay (DIMERTEST STRIPWELL EIA, AGEN Inc., New Jersey, USA.). The method is based on a monoclonal antibody which binds specifically to the D-dimer and fragments containing the D-dimer epitope that result from fibrinolysis. The within batch coefficients of variation across the working range of the assay was <5%.

C-reactive protein; This was measured by Fluorescence Polarisation Immunoassay using an Abbott TDX^{R} analyser and Abbott reagents. The between batch coefficient of variation was <5% across the working range of the assay.

Interleukin-6; Interleukin-6 was determined by bioassay using 7TD1 cells (Coulie et al., 1987). The assay is based on the proliferation of a lymphoid cell line that has a specific dependance on the cytokine for growth. The limit of detection for interleukin-6 was approximately 10pg/ml.

Cortisol; Cortisol was measured by an in-house radioimmunoassay (Glasgow Royal Infirmary) using antisera obtained from the Scottish Antibody Production Unit (Carluke, Scotland). Separation of the assay was performed using a solid phase technique. The detection limit of the assay was 40nmol/l and the between batch coefficient of variation was <10% across the working range of the assay.

Insulin; Insulin concentrations were analysed by a radioimmunoassay using sepharose covalently linked to a second antibody as the separation system. The between batch coefficent of variation was 10.5% at a concentration of 30mU/l.

Glucose; Glucose was analysed by an enzymatic colorimetric method using glucose oxidase (Hitachi 737 Autoanalyser).

Amino acids; The concentrations of free amino acids in the plasma were determined by high performance liquid chromatography using o-phthaldialdehyde/3 mercaptopropionic acid as the derivatisation agent and employing a 3um particle size reversed-phase column (Graser et al., 1985).

3.5.3 Tissue analyses

R.N.A.; R.N.A. was analysed by U.V. absorption, at 260nm, of the supernatant following acid extraction from tissue (dry weight 20mg) with correction for the peptide content of the perchloric acid extract (Fleck and Begg, 1965). The procedure was as follows; 20mg freeze dried tissue (liver or muscle) was weighed out into a glass tube. 1ml of ice-cold 0.2mol/l perchloric acid was then added and mixed. On standing for 10min, the sample was then centrifuged and the supernatant removed. The sample was then washed twice with 1ml 0.2mol/l perchloric acid. The excess acid was drained by inverting the tube over filter paper. The precipitated

material was then incubated for 1h in 0.8ml 0.3mol/l KOH at 37°C and 2x20ul samples were removed for protein determination (see protein below). The tube was then cooled in ice and 0.6ml 1mol/l perchloric acid added and stood in ice for 10min. The sample was then centrifuged, supernatant collected and the precipitate was washed twice more with 1ml 0.2mol/l perchloric acid. The supernatant and washings were mixed together and 6.6ml water added to give a final concentration of 0.1M perchloric acid. The absorbances of the reagent blank (0.8ml 0.3mol/l KOH + 0.6ml 1mol/l perchloric acid + 2ml 0.2mol/l perchloric acid + 6.6ml water) and samples were measured at 260nm and 232nm.

The main source of error in estimating RNA by this method is the presence of U.V.-absorbing peptides in the RNA extract (Munro and Fleck, 1969). It has been demonstrated that in muscle samples there is a greater amount of U.V. absorbing peptides in the RNA fraction compared with liver samples. In liver samples the peptide content of the RNA extract, on average, accounts for 2% of the total U.V. absorption at 260nm, whereas in muscle samples it accounts for 10% (Munro and Gray, 1969). This is mainly due to the fact that the amount of RNA present in the muscle samples is small and in consequence absorption due to peptide content becomes significant (Munro and Fleck, 1969). Therefore, in order to detect and correct for the presence of peptides in liver and muscle RNA extracts (Figure 3.4) absorption measurements were taken at 232nm as well as 260nm (DU7 spectrophotometer, Beckman Instruments, High Wycombe, UK.) and the following equations were applied (Fleck and Begg, 1965).

For liver, $C_{RNA} = 3.40_{E260} - 1.44_{E232}$

For muscle, $C_{RNA} = 3.79_{E260} - 1.50_{E232}$

Where C_{RNA} is the concentration in ugRNA phosphorus per ml, E is the absorbance measured at the defined wavelength. It should be noted

that the equation for muscle was derived from work on calf thyroid (Fleck and Begg, 1965), however, the contribution of peptide material to the absorption of RNA at 260nm was similar (11%) to that reported for muscle (10%, Munro and Gray, 1969). Therefore, although this approach has not been validated specifically for muscle RNA extracts it has been used to measure the muscle RNA content in man (Rennie et al., 1982a; Emery et al., 1984a; Gibson et al., 1987; Forsberg et al., 1991).

Repeated analysis (n=5) of a normal human liver biopsy established the within batch variation of the assay to be 8%.

Protein; The protein concentration in the alkali-soluble material from the RNA method (see above) was measured by the method of Lowry and coworkers (1951). The procedure was as follows; the protein sample was diluted 1/20 with water. Biuret reagent was made from 100ml 2%$NaCO_3$ in 0.1mol/l NaOH (w/v), 1ml 2% NaK tartrate (w/v), 1ml 1% $CuSO_4$ (w/v, BDH, U.K.). A working standard curve in the range 0-50ug was prepared by taking volumes (0-50ul) of a stock solution (1mg/ml human albumin, Fraction V, A8763, Sigma Chemical Co., U.K.) and adjusting the final volume to 400ul with water. To 400ul standard/sample 2ml Biuret reagent was added, vortexed and allowed to stand for 10min. 200ul of working Folin-Ciocalteau reagent (water 1:1 Folin-Ciocalteau reagent, BDH, U.K.) was added with immediate mixing. The solution was allowed to stand for 30min and the absorbance was measured at 750nm (DU7 spectrophotometer, Beckman Instruments, High Wycombe, UK.) and the concentrations were read from the std curve).

Tissue free amino acids; The concentrations of free amino acids in liver and muscle tissue were determined by high performance liquid chromatography using o-phthaldialdehyde/3 mercaptopropionic acid as the

derivatisation agent and employing a 3um particle size reversed-phase column (Graser et al., 1985).

Protein electrophoresis; Proteins were electrophoresed on an agarose film (Ciba Corning Diagnostics, CA, USA.) using universal barbital buffer, (Ciba Corning Diagnostics, CA, USA.) immunofixed and stained with Amido Black 10B (Ciba Corning Diagnostics, CA, USA.). Electrophoresis is based on the principle that a charged ion or group will migrate towards one of the electrodes when placed in an electric field (Smith, 1968). This technique has been used extensively to separate proteins. Immunofixation allows specific identification of protein in the same position as stained in the electrophoresed material and involves using an immunological overlay to fix the specific protein. A serum quality control sample (Seronorm, Nycomed, UK.) was run with every electrophoresis film. 1ul of the acid/alcohol extract (see section 3.4.2) concentrated to 150ul or control sample (plasma sample diluted 1:10) was applied to the sample wells of the agarose film. The film was then inserted into a cassette and placed in an electrophoresis chamber (Ciba Corning Diagnostics, CA, USA.), each cell of which contained 95ml of barbitone buffer. The film was run for 27min and removed from the cassette. Strips of cellulose acetate were placed over the area of the film containing samples to be immunofixed. To these samples 25ul of antiserum (to albumin or fibrinogen, Scottish Antibody Production Unit) was applied to the strips. The film was incubated in a moist chamber for 2-4h at room temperature and then placed in 1l normal saline overnight to remove non-precipitated proteins. Films with immunofixed material and the conventional electrophoresed material were then stained with Amido Black 10B working solution for 10min. The films were then dried, cleared in two

changes of 5% acetic acid, dried and scanned in a densitometer (Model 620 Video Densitometer, Biorad laboratories Inc, U.K.).

3.6 Resting energy expenditure measurement

3.6.1 Introduction

The measurement of energy expenditure is referred to as calorimetry in which energy expended is measured as heat output. Energy can neither be created nor destroyed and therefore the energy content of any system can be increased or decreased only by the amount of energy that is added to or lost from the system. Measurements of heat associated with the metabolism of man and animals, have long been of scientific interest. Both measurements of heat loss (direct calorimetry) and estimates of heat production by measuring gas exchange (indirect calorimetry) were first made approximately two centuries ago (Kinney, 1988).

3.6.2 Direct calorimetry

Man and animals are constantly producing heat from the chemical reactions in the body necessary to maintain normal metabolism. As this energy is not stored in the body, where it would increase body temperature to unacceptable levels, it must be lost to the environment. Direct calorimetry, the measurement of this heat loss, can be carried out in various ways, the accuracy of which depends only on the physical techniques used. No assumptions about the means of production need be made and the subject can be treated as a 'black box' producing heat. Since all direct calorimeters have to totally enclose the subject to measure heat loss, they are generally unsuitable for subjects who need frequent attention (e.g. patients, children etc.).

3.6.3 Indirect calorimetry

Production of heat from food or tissue fuels in the body consumes oxygen and produces carbon dioxide and other excretory products. Measurement of gas exchange and urinary nitrogen excretion provides an accurate method of indirect calorimetry when metabolic processes are in approximate equilibrium and gives the same values as measured by direct calorimetry (Kinney, 1988). This technique has been used, over the last 10 years, to measure energy expenditure in hospitalised patients in the Department of Surgery, Glasgow Royal Infirmary (Goll, 1981; Hansell, 1986). This indirect calorimetry system was used to estimate energy expenditure in the patients studied in this work.

3.6.4 Resting energy expenditure

Energy expenditure varies according to the activity of the subject; being about 20% above resting levels when a human subject is sitting and 50% when standing (Durnin and Passmore, 1967). Therefore, control and consistency in physical conditions of calorimetric measurement are essential. The term 'basal metabolic rate' has been used by many authors (Dubois, 1927; Keys et al., 1950) to describe energy expenditure measurement on a quiet, resting, post-absorptive subject. Even if the conditions are more strictly standardised to include a thermoneutral environmental temperature, complete mental and bodily rest and a measurement period just after waking, the metabolic rate is unlikely to be a basal value proposed by Mitchell (1962). Body temperature, previous nutrition and sleep patterns are at least three of the variables that can effect it (Buskirk et al., 1960).

Energy expenditure measured with the subject lying down at rest and having no recent large meals has been termed resting energy

expenditure. Measurements made in this manner are usually close to the basal values described above (Durnin and Passmore, 1967), while allowing a wider experimental scope. The respiratory quotient (RQ) in such circumstances is not necessarily well defined. However, control of the nutritional input, particularly if it is small or constant, can allow useful comparisons. To decrease the variability of resting energy expenditure values environmental conditions should be as constant as possible. Psychological stress has been shown to have a large effect in man (Arturson, 1977). This can be difficult to detect and impossible to quantify but can be mostly eliminated by use of a comfortable and relatively isolated environment. Training of the human subject in the use of the calorimetry apparatus is also important (Robertson and Reid, 1952) particularly where a mask or mouthpiece is utilised (Kinney et al., 1964). Ingestion of significant quantities of food increases metabolic rate (Kleiber, 1961). The rise in metabolic rate following a meal starts almost immediately (Passmore and Ritchie, 1957) with the peak resting energy expenditure usually within two hours (Rochelle and Horvath, 1969). Sustained increases in oxygen consumption of up to 20% often occur (Tuttle et al., 1953) so that control over dietary intake is necessary if small changes in resting energy expenditure are to be measured.

3.6.5 Indirect calorimetry technique

With the advent, in the 1950's, of continuous gas flow measurement of oxygen and carbon dioxide concentrations continuous indirect calorimetry has been possible. Using a suitable mouthpiece or mask and one way valve, expired air can be analysed, breath by breath or after mixing (Wilmore, Davis and Norton, 1976). This technique has the advantage of relatively high (approximately 5%) changes in gas

concentrations produced by the subject allowing accurate analysis with the possibility of measuring other respiratory parameters. However, rapid sampling or effective mixing of the gas flow is required and the relatively low (approximately 5l/min) pulsatile flow can be difficult to measure accurately. Furthermore the valving provides some resistance to air flow and patients can also find mouthpieces and facemasks unacceptable (Spencer et al., 1972).

The use of an open or closed canopy system (ventilated hood system) instead of a mouthpiece or mask requires a relatively high air flow (25-50l/min) to be drawn through the canopy to keep the carbon dioxide concentration around the patient to within acceptable physiological limits (i.e. less than 1%). The diluting effect of the increased air flow requires high accuracy of the gas analysers. However, the subject in the canopy notices no effect on his breathing and the constant air flow through the system can be measured with a simple gas meter. The indirect calorimeter used in the present studies was of a closed canopy type based on the design of Kinney and coworkers (1964). A detailed description of the indirect calorimeter and its validation for use in patient studies is given by Goll (1981). A schematic representation of the gas circuitry is shown in Figure 3.5.

A sensitive paramagnetic oxygen analyser (Servomex Ltd, Crowborough, Sussex, UK) and an infra-red carbon dioxide analyser (Sieger Ltd, Poole, Dorset, UK) were used to monitor oxygen and carbon dioxide gases respectively. The equipment was calibrated frequently using oxygen-free nitrogen, 0.80% carbon dioxide and air at a known barometric pressure. All the measurements were made under computer control. The readings were corrected for atmospheric pressure and in the case of the air used as a span gas for the oxygen analyser for its moisture content. The

computer calculated correction factors, close to unity, to account for the long term gain drift in the analysers. Gain and zero drifts of the instruments were not corrected using the various mechanical and electronic adjustments available, but the correction factor was applied by the computer during the calculation of results. This approach improved long term stability of the analysers as it resulted in less interference and interdependent effects which make accurate setting difficult (e.g. a change in gain on adjustment of the zero point were avoided).

The sensitivity and accuracy of the calorimeter was checked periodically by burning a known weight of fuel, in the canopy, to simulate a subject (Caldwell et al., 1966; Kinney et al., 1968). Butane was selected as an appropriate fuel with a 'respiratory quotient' of 0.615. Commercial butane gas (Camping Gaz Ltd.) in a cartridge was burnt in a small luminaire designed for auxillary lighting purposes (Lumogaz, Camping Gaz Ltd.). The apparatus was self-contained and could fit inside the head canopy. From analysis of the gas composition and the amount of oxygen consumed and carbon dioxide produced per unit weight of gas the consumption of oxygen and production of carbon dioxide was calculated. The oxygen consumption was 97.2% and carbon dioxide production was 97.4% of the calculated value.

Resting energy expenditure was measured using the open circuit ventilated hood system, described above, in a temperature controlled room. The subject's head was enclosed in the perspex canopy and the system made air tight by a flexible adhesive neck seal. The canopy was ventilated with air at a rate of 35-40l/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 1min. The whole system provides measurements of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) for which the repeatability is better than $\pm$5% (95% confidence intervals). Estimates of VO_2 and VCO_2 were collected every 30 seconds during each patient study, which lasted for 40min. Recording of data did not commence until a steady trace of oxygen consumption and carbon dioxide production was obtained, usually after 5-10 min. The 80 estimates of VO_2 and VCO_2 collected were processed on line by a programmed microcomputer (Goll, 1981) and converted to mean energy production (watts) using the abbreviated formula of Weir (1949).

$$\text{REE (kcal/day)} = (3.9VO_2 + 1.1VCO_2)1440$$

$$\text{Where kcal/day} = \text{wattsx}20.65$$

$$VO_2 = \text{oxygen consumption (l/min)}$$

$$VCO_2 = \text{carbon dioxide production (l/min)}$$

Since resting energy expenditure is known to vary with the size of the individual (Mitchell, 1962) it is necessary to express energy expenditure in relation to total body weight or a defined compartment of the body to allow comparison of results from subjects of different height and weight. Durnin (1959) suggested that use of weight alone was a good enough reference standard. However, this neglects differences in build and it has been reported that there is a better association between lean body mass and resting energy expenditure (Halliday et al., 1979). However, lean body mass was not measured in this work and therefore, resting energy expenditure was expressed per unit weight.

Table 3.1

Recovery of plasma [14C]glycine after sample preparation procedures

	Ultra-filter	Cation-exchange	HPLC	Overall recovery
	(% recovery of procedure)			(%)
Sample 1	88.1	85.4	80.9	60.9
2	90.4	88.9	91.2	73.3
3	94.1	94.6	91.2	81.2
4	89.3	88.7	89.5	70.9
5	86.0	89.1	95.4	73.1
Mean	89.6	89.3	89.6	71.9
S.D.	3.0	3.3	5.3	7.3

Ultra-filter, ultrafiltration; HPLC, high performance liquid chromatography of amino acids

The overall recovery for the sample preparation was approximately 70%.

Table 3.2

Recovery of plasma free amino acids after ultrafiltration

	Plasma (umol/l)	Ultrafiltered plasma (umol/l)	% RECOVERY
ASP	31	29	95
GLU	303	300	99
ASN	31	28	98
SER	161	145	90
GLN	173	149	86
GLY	230	209	91
THR	113	96	85
HIS	111	92	83
CIT	37	36	99
3-MH	5	4	80
ALA	284	239	84
ABU	22	18	80
TYR	49	37	76
VAL	203	179	88
MET	21	19	91
TRP	33	30	91
PHE	64	56	87
ILE	77	62	81
LEU	129	103	80
ORN	58	34	58
LYS	111	60	54
TOTAL AA	2349	1932	83

Table 3.3

The effect of temperature and pyridine formate concentration on amino acid separation

SAMPLE	F.R. (ml/min)	P.F. (MOL/L)	TEMP. (oC)	Retention time (min) GLU	ALA	GLY	VAL	ILE	LEU	Comments
AAMIX	0.4	0.1	40	51.2	62	72.8	82.8	114.4	125	Complete separation of
AAMIX	0.4	0.1	40	46.8	56.4	66.4	75.4	104	114	glycine and leucine from
AAMIX	0.4	0.1	40	52.4	63.2	74.4	84.4	116.8	127.6	other amino acids
AAMIX	0.4	0.1	40	50.8	61.2	72	81.6	112.8	123.6	
AAMIX	0.4	0.1	50	43.6	52.8	62	70.4	97.2	106.4	Complete separation of
AAMIX	0.4	0.1	50	42	51.2	60	68	94	102.8	glycine and leucine from
AAMIX	0.4	0.1	50	42	51.2	60	68	94	102.8	other amino acids
AAMIX	0.4	0.1	50	44.8	54.4	64	72.4	100.4	109.6	
AAMIX	0.4	0.1	50	42.4	51.6	60.8	68.8	95.2	104.4	
AAMIX	0.4	0.15	50	30.4	36.8	43.2	49.2	68	74.4	Complete separation of
AAMIX	0.4	0.15	50	30	36.4	42.8	48.8	67.2	73.6	glycine and leucine from
AAMIX	0.4	0.15	50	30	36.4	42.8	48.8	67.2	73.6	other amino acids
AAMIX	0.4	0.15	50	30	36.8	45.6	52	71.6	78.4	
AAMIX	0.4	0.15	50	32	38.8	45.6	52	71.6	78.4	
AAMIX	0.4	0.2	40	24.8	30	35.2	40	55.2	60.4	Complete separation of
AAMIX	0.4	0.2	40	24.8	30	35.2	40	55.2	60.4	glycine and leucine from
AAMIX	0.4	0.2	40	24.8	30	35.6	40	55.6	60.8	other amino acids
AAMIX	0.4	0.2	40	24.8	30	35.6	40	55.6	60.8	
AAMIX	0.4	0.2	40	24.8	30	35.6	40	55.6	60.8	
AAMIX	0.4	0.2	40	25.6	31.2	36.4	41.2	57.2	62.4	
AAMIX	0.4	0.2	40	25.6	31.2	36.4	41.2	57.2	62.4	
AAMIX	0.4	0.2	40	25.2	30.4	35.6	40.4	55.6	61.2	
AAMIX	0.4	0.2	40	25.2	30.4	36	40.8	56.4	61.6	
AAMIX	0.4	0.2	40	24.8	30	35.2	40	55.2	60.4	
AAMIX	0.4	0.2	40	25.2	30	35.6	40.4	55.6	61.2	
AAMIX	0.4	0.2	50	20.8	25.2	29.6	33.6	46.4	50.8	Complete separation of
AAMIX	0.4	0.2	50	20.4	24.8	29.2	33.2	45.6	50	glycine and leucine from
AAMIX	0.4	0.2	50	20.8	25.2	29.6	33.6	46.4	50.8	other amino acids
AAMIX	0.4	0.2	50	20.4	24.8	29.2	33.2	45.6	50	
AAMIX	0.4	0.2	50	20.8	25.2	29.6	33.6	46.4	50.8	
AAMIX	0.4	0.2	50	20.4	24.8	29.2	33.2	45.6	50	
AAMIX	0.4	0.2	50	20.4	24.8	29.2	33.2	45.6	50	
AAMIX	0.4	0.2	50	17	20.8	24.4	27.6	38	41.6	
AAMIX	0.4	0.2	50	17.2	21.2	24.4	28	38.8	42.4	
AAMIX	0.4	0.2	50	19.2	22.8	27.2	30.8	42.4	46.4	
AAMIX	0.4	0.2	50	21.6	26	30.8	34.8	48.4	52.8	
AAMIX	0.4	0.2	50	21.6	26	30.8	34.8	48.4	52.8	
AAMIX	0.4	0.2	50	21.6	26	30.8	34.8	48.4	52.8	
AAMIX	0.4	0.2	50	21.6	26.4	30.8	35.2	48.4	53.2	
AAMIX	0.4	0.2	50	22.4	26.8	31.6	36	49.6	54.4	
AAMIX	0.4	0.2	50	19.2	23.2	27.6	31.2	43.2	47.2	
AAMIX	0.4	0.2	50	19.6	23.6	28	31.6	43.6	48	
AAMIX	0.4	0.2	50	19.6	23.6	28	31.6	43.6	48	
AAMIX	0.4	0.2	60	19.2	23.2	27.2	30.8	42.4	46.4	Complete separation of
AAMIX	0.4	0.2	60	19.2	23.2	27.2	30.8	42.4	46.4	glycine and leucine from
AAMIX	0.4	0.2	60	18.8	22.8	26.8	30.4	41.6	45.6	other amino acids
AAMIX	0.4	0.2	60	18.4	22	26	29.6	40.8	44.4	
AAMIX	0.4	0.2	60	18.4	22.4	26.4	29.6	41.2	45.2	
AAMIX	0.4	0.2	60	19.2	23.2	27.2	30.8	42.4	46.4	
AAMIX	0.4	0.2	60	19.2	23.6	27.6	31.2	43.2	47.2	
AAMIX	0.4	0.2	60	19.2	23.6	27.6	31.2	43.2	47.2	
AAMIX	0.4	0.2	60	19.2	23.6	27.6	31.2	43.2	47.2	
AAMIX	0.4	0.2	60	19.2	23.6	27.2	31.2	43.2	47.2	
AAMIX	0.4	0.2	60	19.2	23.2	26.8	30.8	42.4	46.4	
AAMIX	0.4	0.2	60	18.8	22.8	26.8	30.4	42	46	
AAMIX	0.4	0.2	60	19.2	23.2	27.2	30.8	42.4	46.4	
AAMIX	0.4	0.2	60	18.8	22.8	26.8	30.4	41.6	45.6	
AAMIX	0.4	0.25	50	13.2	16	19.2	21.6	30	32.8	Incomplete separation of
AAMIX	0.4	0.25	50	14	16.8	19.6	22.4	31.2	34	glycine and leucine from
AAMIX	0.4	0.25	50	14	16.8	19.6	22.4	31.2	34	other amino acids
AAMIX	0.4	0.25	50	14	16.8	19.6	22.4	31.2	34	
AAMIX	0.4	0.25	50	14	16.8	19.6	22.4	31.2	34	
AAMIX	0.4	0.25	50	15.6	18.8	22	25.2	34.8	38.4	
AAMIX	0.4	0.25	50	15.2	18.4	22	24.8	34.4	37.6	

The effect of different concentrations of pyridine formate on retention time. The purity of the amino acids was determined as described in section 3.3.4. F.R. flow rate, P.F. pyridine formate, TEMP. temperature, GLU glutamic acid, GLY glycine, Val valine, ILE isoleucine, Leu leucine.

Table 3.4

Amino acid separation of plasma using pyridine formate

time (min)	DPM	DPM	
0-2.5	26	30	
2.5-5.0	27	44	
5.0-7.5	22	71	
7.5-10	37	63	
10.0-12.5	10	48	
12.5-15.0	55	23	
15.0-17.5	18	38	
17.5-20.0	28	30	
20.0-22.5	106	78	
22.5-25.0	4625	7903	
25.0-27.5	15006	19599	alanine
27.5-30	3992	4625	
30.0-32.5	10270	11498	glycine
32.5-35	11	30	
35.0-37.5	20	26	
37.5-40	33	38	
40.0-42.5	129	966	
42.5-45	34061	26423	
45.0-47.5	41565	35072	isoleucine
47.5-50	3927	4670	
50.0-52.5	15429	13971	leucine
52.5-55.0	1208	391	
55.0-57.5	7	10	

time (min)	DPM	DPM	DPM	DPM	
20.0-20.5	9	15	8	16	
20.5-21.0	11	11	13	14	
21.0-21.5	10	10	10	11	
21.5-22.0	13	12	9	10	
22.0-22.5	4	11	12	15	
22.5-23.0	14	12	18	17	
23.0-23.5	19	18	19	24	
23.5-24.0	43	26	31	51	
24.0-24.5	519	671	490	820	
24.5-25.0	2485	7700	14173	9111	
25.0-25.5	4410	11954	26635	17251	alanine
25.5-26.0	4089	8278	7303	16227	
26.0-26.5	1960	5347	289	9329	
26.5-27.0	205	143	11	1520	
27.0-27.5	63	28	7	462	
27.5-28.0	30	424	28	30	
28.0-28.5	637	3129	472	31	
28.5-29.0	2580	9403	13887	1520	
29.0-29.5	6183	13942	26688	6742	
29.5-30.0	5234	10556	7527	18900	glycine
30.0-30.5	2453	6567	233	9012	
30.5-31.0	268	1121	7	1314	
31.0-31.5	79	127	7	209	
31.5-32.0	27	20	11	19	
32.0-32.5	19	17	12	16	
32.5-33.0	18	15	15	15	
42.0-42.5	2	10	17	6	
42.5-43.0	8	7	5	1	
43.0-43.5	13	5	19	26	
43.5-44.0	7	7	18	7	
44.0-44.5	169	21	99	62	
44.5-45.0	1815	1401	1659	957	
45.5-46.0	8613	15285	48087	28204	
46.0-46.5	14874	24901	90774	53003	isoleucine
46.5-47.0	14401	17500	24881	14325	
47.0-47.5	6702	11159	985	557	
47.5-48.0	691	298	32	21	
48.0-48.5	31	18	19	13	
48.5-49.0	12	44	95	55	
49.0-49.5	1914	6436	1601	993	
49.5-50.0	8702	19042	47212	27536	
50.5-51.0	21481	29124	90929	51309	leucine
51.0-51.5	17655	22012	25619	14798	
51.5-52.0	8362	13718	10793	466	
52.0-52.5	971	2941	213	13	
52.5-53.0	35	265	23	14	
53.0-53.5	26	5	15	21	
53.5-54.0	11	17	19	22	
54.0-54.5	11	11	9	19	
54.5-55.0	12	9	13	12	

Separation of [14C]labelled amino acids (alanine, glycine, isoleucine and leucine) from plasma (see section 3.4.3, 3.4.5). Fractions were collected by time, 2.5min or 0.5min.

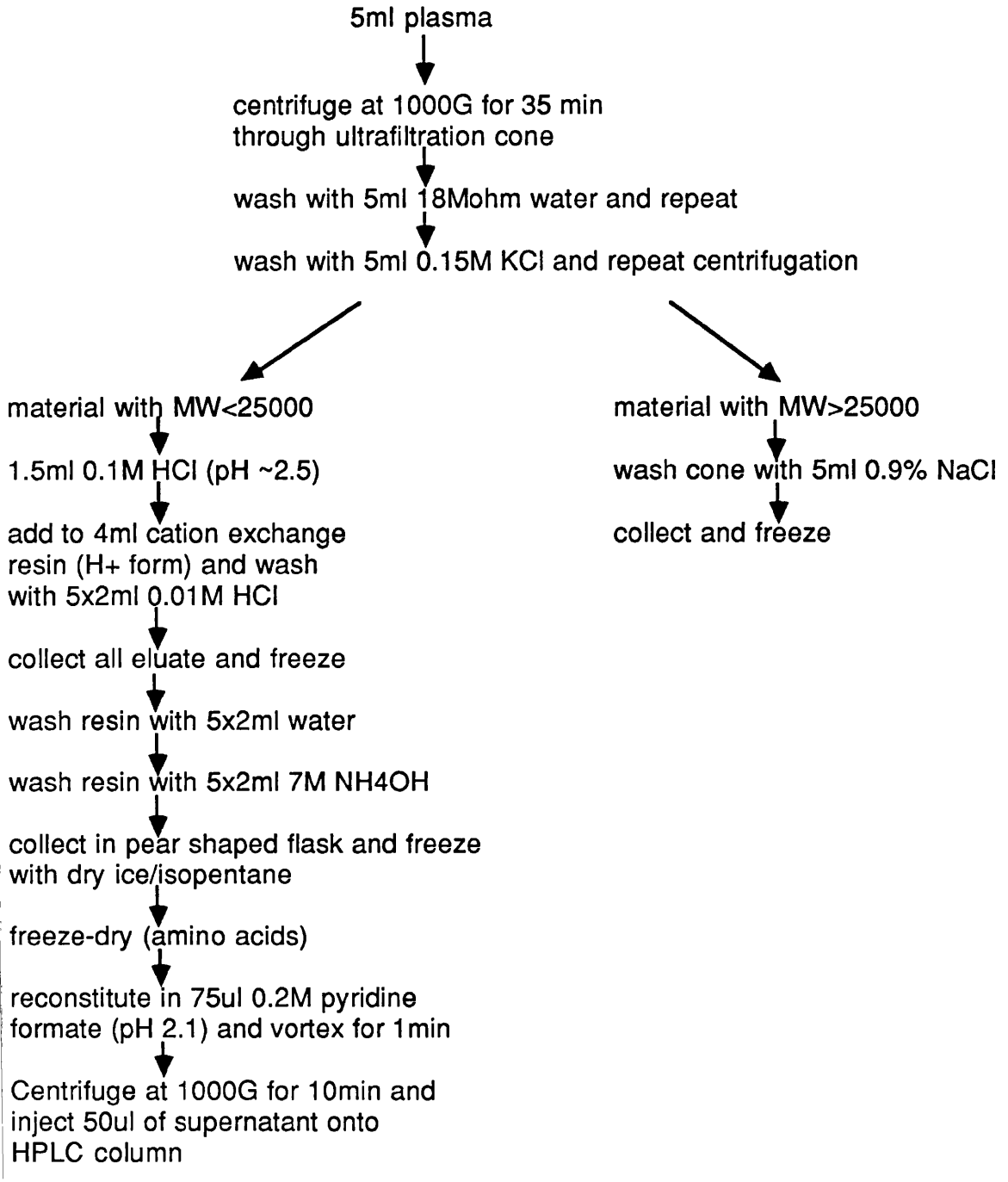


Figure 3.1 Plasma sample preparation

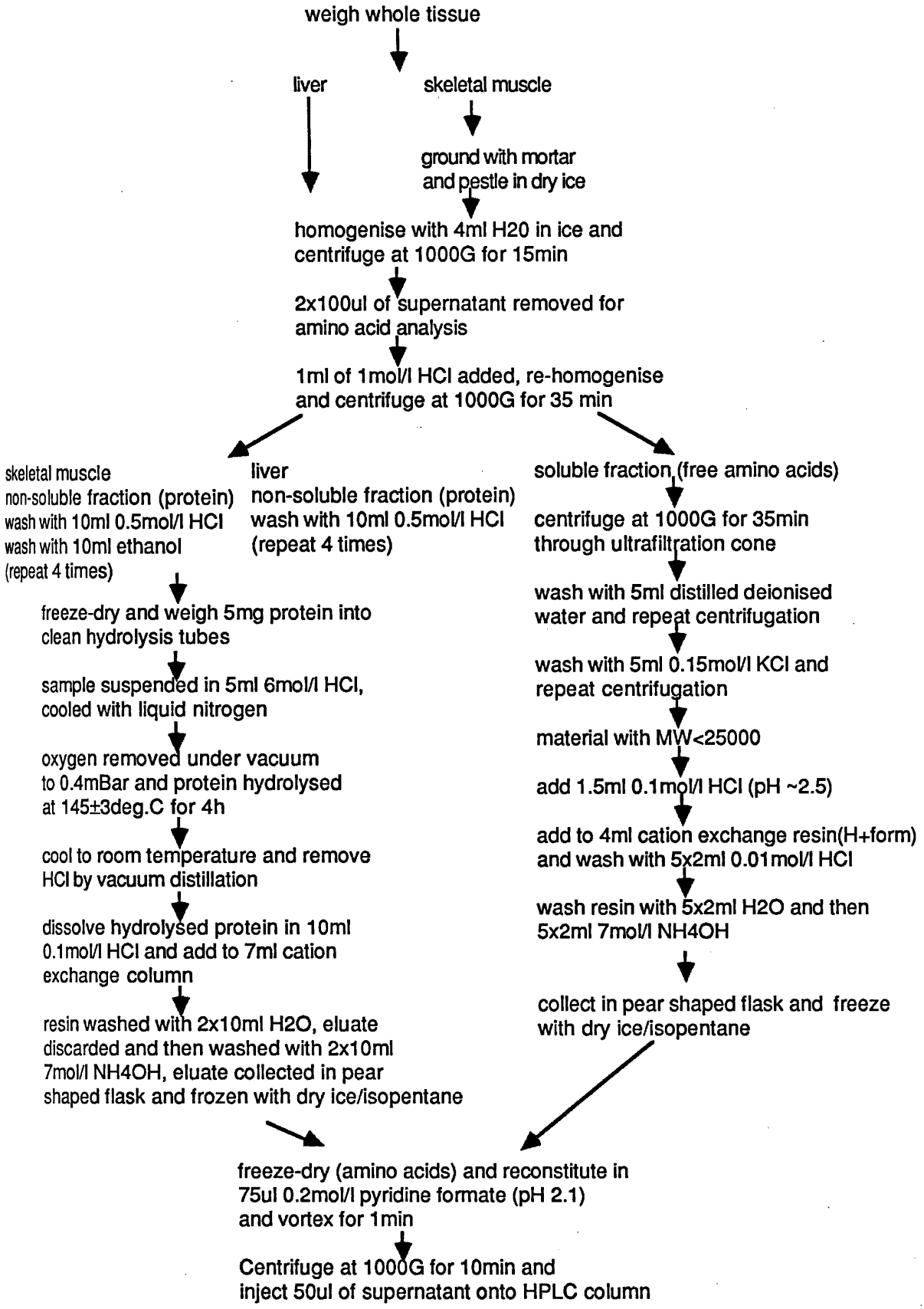


Figure 3.2 Tissue sample preparation

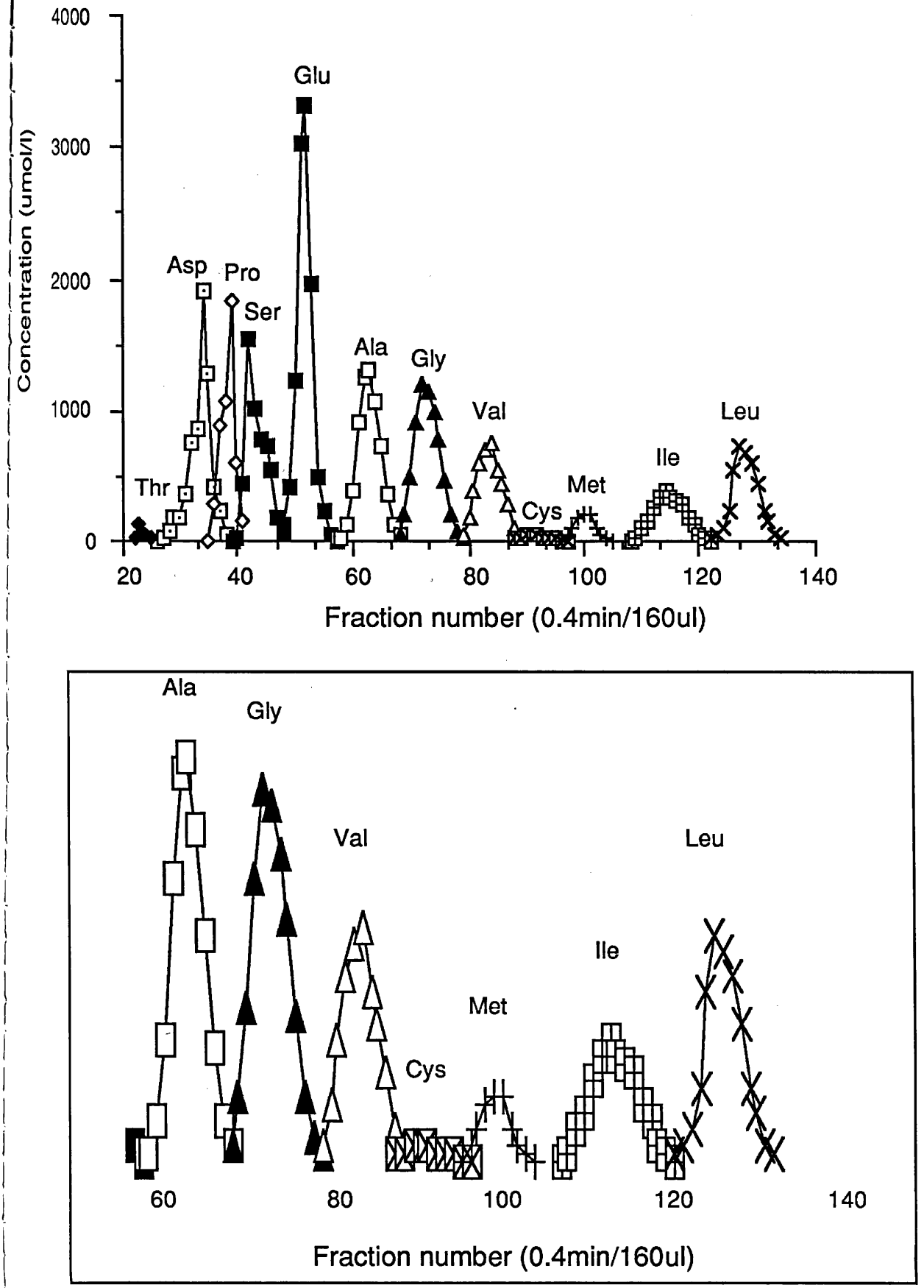


Figure 3.3 . Amino acid separation of liver hydrolysate using 0.2 mol/l pyridine formate. Individual fractions were analysed on a Chromaspek M amino acid analyser. ☐ The expansion of the region of the fractions 60-140 to illustrate the separation of alanine/glycine and isoleucine/leucine.

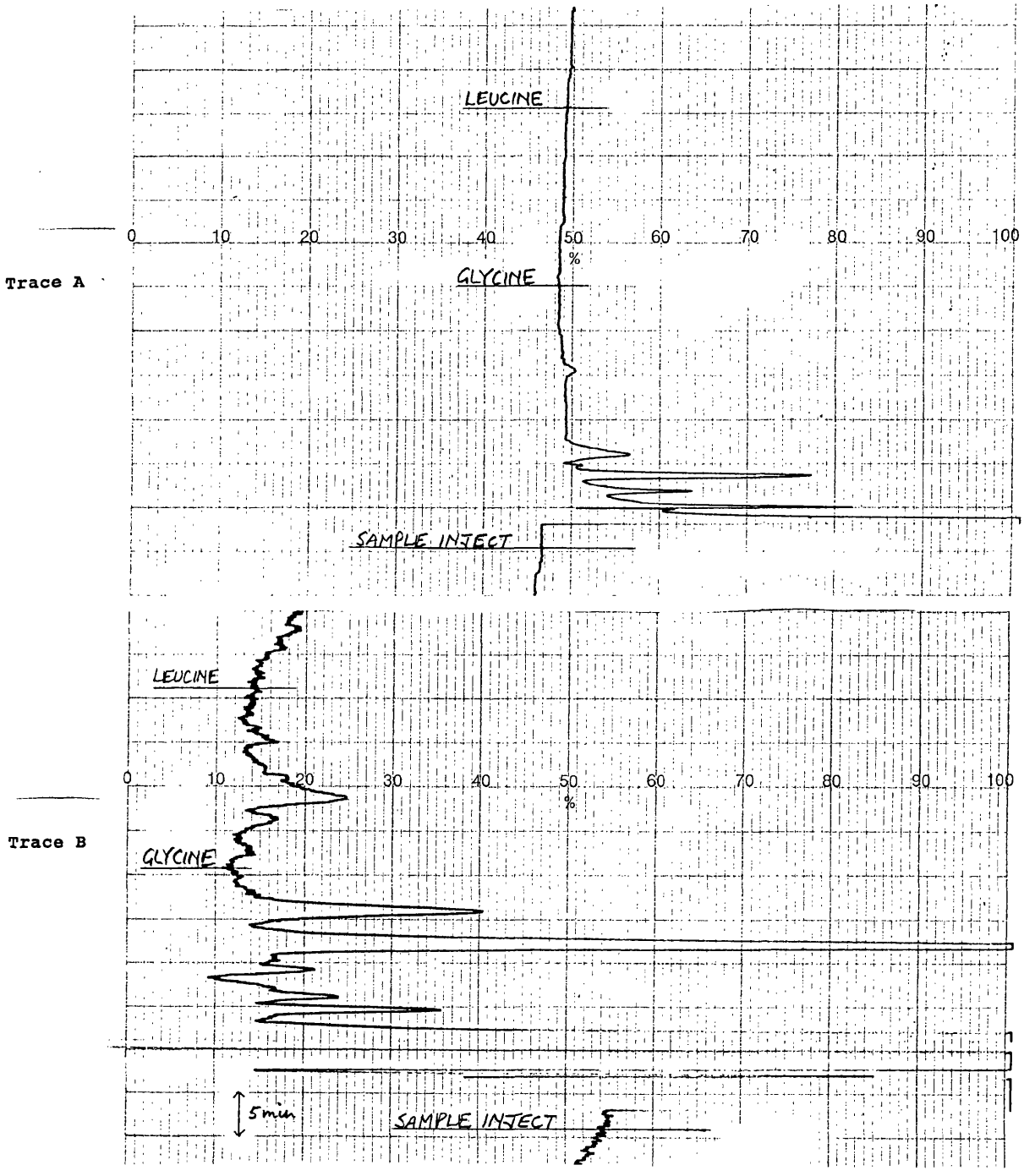


Figure 3.4 U.V. detector trace of amino acid separation.
Traces A and B show plasma amino acid separation using 0.2mol/l pyridine formate at 50°C at different detector sensitivities, trace A (0.08 Absorbance Units Full Scale) and trace B (0.01 Absorbance Units Full Scale). U.V. detector at 278nm and chart speed 120mm/min. Retention times of glycine and leucine are shown.

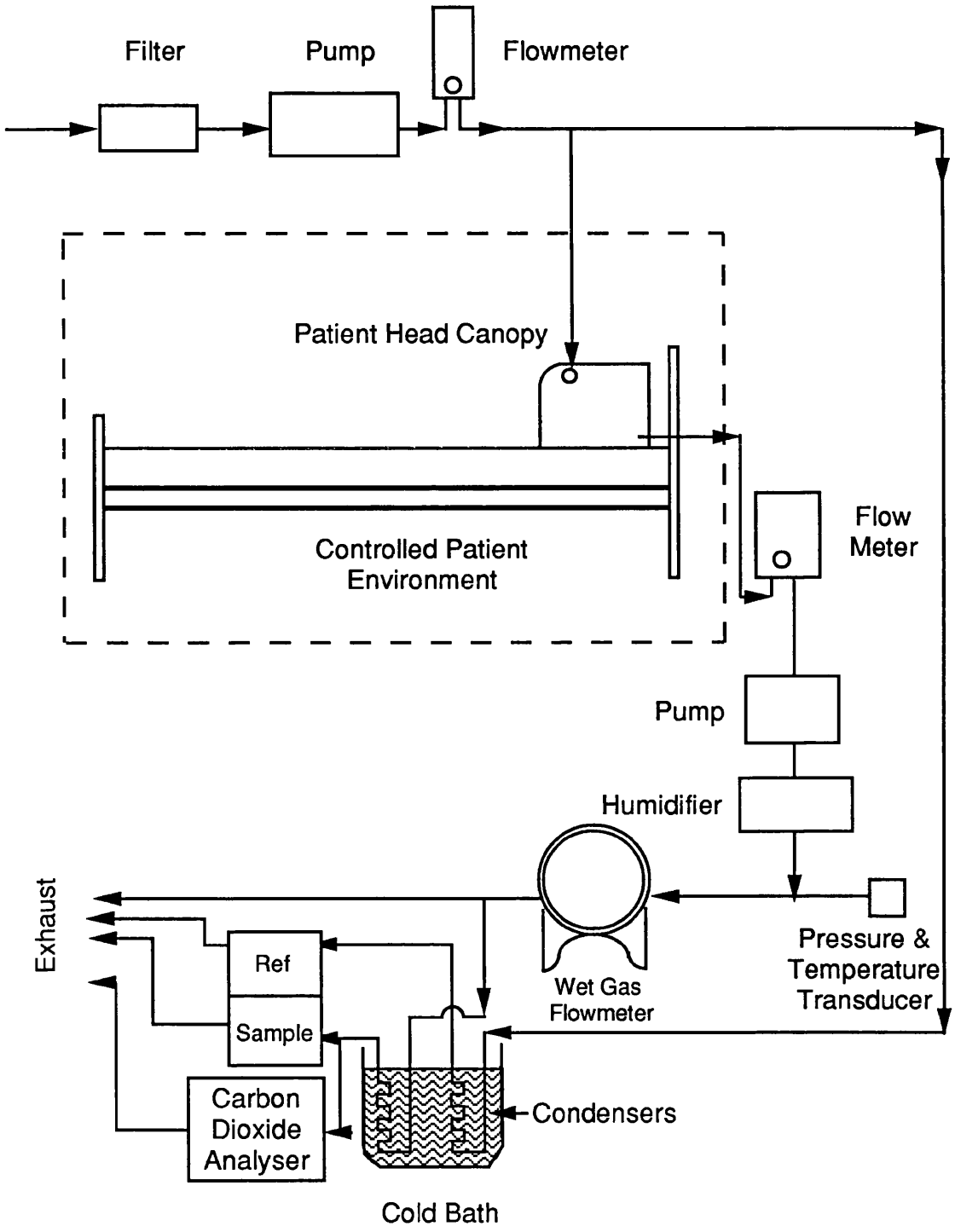


Figure 3.5 Patient indirect calorimeter

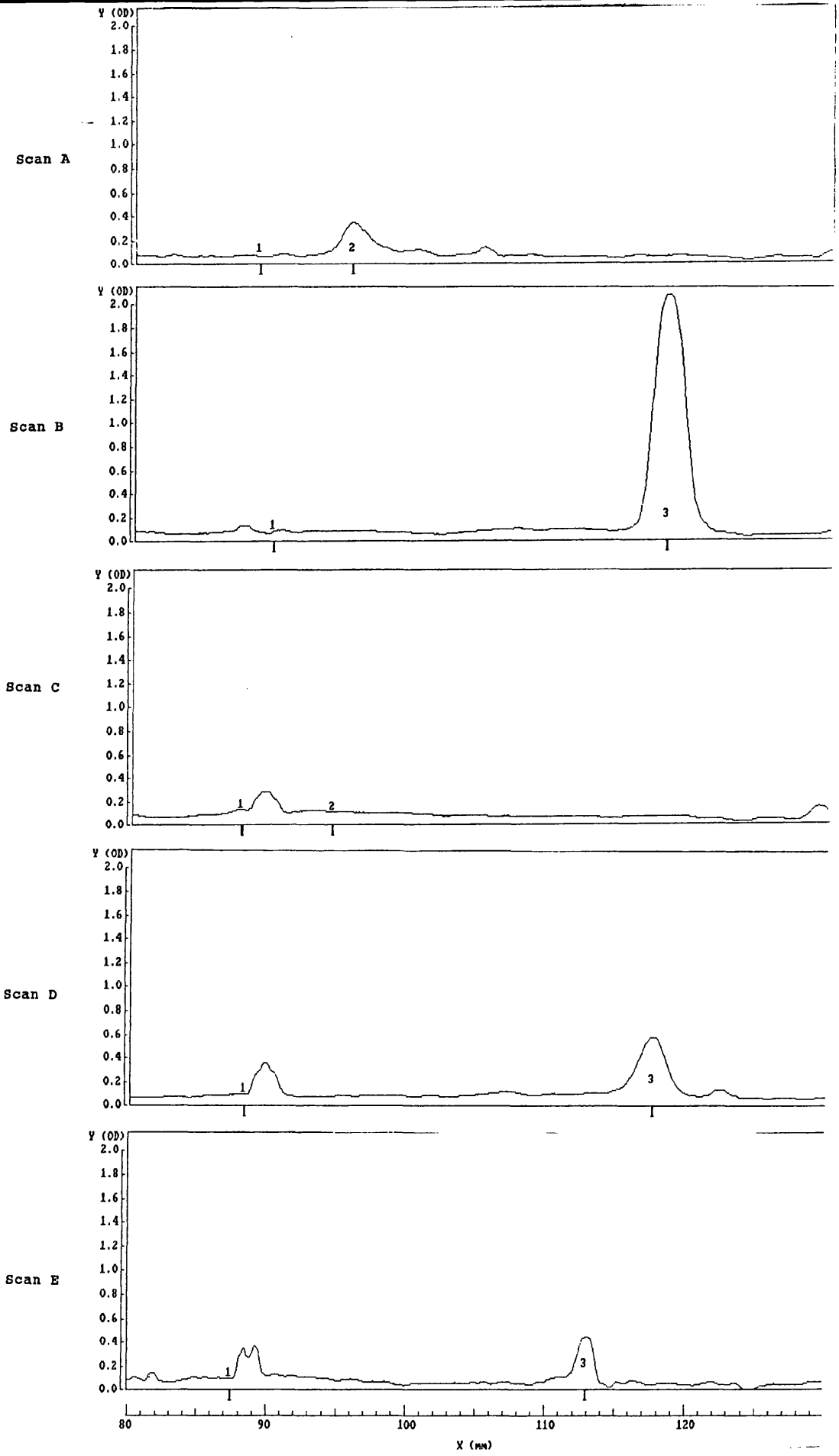


Figure 3.6 Electrophoresis and immunofixation of the acid/alcohol extract from skeletal muscle biopsy (see section 3.5.3). Scan A and B, electrophoresis and immunofixation of plasma (diluted 1:10) using antiserum to fibrinogen (Scan A) and albumin (Scan B). Scan C and D, electrophoresis and immunofixation of acid/alcohol extract using antiserum to fibrinogen (Scan C) and albumin (Scan D). Scan E, electrophoresis of acid/alcohol extract. 1= sample well, 2= fibrinogen, 3= albumin, X= distance in mm.

CHAPTER 4: METHODS: STABLE ISOTOPE MEASUREMENT AND STUDY PROTOCOL VALIDATION

4.1 Introduction

The stable isotopes of carbon (^{13}C), nitrogen (^{15}N), oxygen (^{18}O) and hydrogen (^{2}H) were all discovered in the period 1929-1932 and soon assumed an important role in biochemical studies. In the absence of radioactive isotopes a wealth of published material appeared on the separation and detection of stable isotopes, the synthesis of labelled compounds, their use in biochemistry, and especially for the study of the metabolism of proteins, carbohydrates and lipids. The subsequent widespread use of radioactive isotopes in the same fields, after the second world war, reduced the use of their stable equivalents which were disadvantaged in terms of isotope cost and the availability and expense of instrumentation. However, since the early 1970's there has been a return to the use of stable isotopes in certain fields for reasons that were discussed in Chapter 2.

The enrichment of stable isotopes can be measured by a number of techniques. However, the precision of isotope analysis required and the sample size available in substrate turnover and incorporation studies means that many of the techniques capable of isotope analysis are not suitable for such studies. In the measurement of whole body and tissue protein synthesis using a tracer dose of labelled amino acid, the analytical technique must be capable of measuring samples which vary in enrichment between natural abundance and approximately 3.0atom% excess. Furthermore, a precision of at least 0.3% (relative to the enrichment) ^{15}N or ^{13}C on samples as small as 1umol of amino acid is required. For

example $\pm$ 0.001 atom% ^{15}N at natural abundance, and $\pm$ 0.01 at 3atom% ^{15}N.

4.1.1 Nuclear Magnetic Resonance

The phenomenon of Nuclear Magnetic Resonance was discovered in 1946 by Bloch and Purcell. Nuclear Magnetic Resonance spectroscopy rapidly became an important technique in organic chemistry for elucidating molecular structures, complementing other techniques such as infrared spectroscopy and mass spectrometry. Certain stable isotopes have a non-zero spin and therefore can be detected by Nuclear Magnetic Resonance spectroscopy. ^{13}C and ^{15}N have nuclear spins of 1/2 and ^{17}O of 5/2. Of these isotopes, the majority of reported work has concerned ^{13}C. ^{13}C nuclei are present in extremely small amounts in most biological systems, and therefore are suitable for use in labelling experiments. For example, the metabolism of ^{13}C-labelled alanine and pyruvate has been followed in a perfused liver system (Cohen, 1983, 1987). There are, however, several disadvantages with this type of application. Firstly, because of the poor sensitivity, of detection of ^{13}C, large concentrations (ie. non-tracer) of labelled precursors must be used, which may distort the metabolic kinetics. Secondly, most ^{13}C compounds are expensive. Thirdly, there is the risk of heating the patient. This is because of the intensive radio frequencies needed to irradiate protons coupled to ^{13}C in order to simplify the spectrum for analysis.

It is clear that Nuclear Magnetic Resonance spectroscopy does not fulfil the criteria stated in the introduction and therefore, is not suitable for the proposed protein synthesis studies.

4.1.2 Emission Spectroscopy

Optical emission spectroscopy is routinely used for the determination of nitrogen-15. The method depends upon the wavelength separation of the isotopic species of nitrogen gas ($^{14}N_2$, $^{14}N^{15}N$, and $^{15}N_2$) due to the isotopic shift. Prior to assay, it is necessary to convert the nitrogen to nitrogen gas. For example, amino compounds are treated with hypobromite. Although compared to the isotope ratio mass spectrometer, more tracer is required for results of comparable accuracy, it has been used to determine urinary ^{15}N-urea, ^{15}N-ammonia and ^{15}N-labelled amino acid on infusion of ^{15}N-glycine into human subjects (Stein et al., 1975). Automated instruments based on this concept have been described (Klein and Klein, 1975). Such optical emission spectrometers have been used in whole body protein turnover studies using [^{15}N]glycine (Stein et al., 1983). However, the analytical precision is not good enough to measure the incorporation of a labelled amino acid into protein pools with a slow turnover (eg. muscle). Furthermore, since carbon isotope ratios cannot be measured by this technique it was not suitable for the studies proposed in this thesis.

4.1.3 Infrared spectroscopy

This technique has limited applications in the detection and assay of labelled compounds. Absorption peaks at 2193 cm^{-1} for ^{12}CO (carbon monoxide) and at 2144 cm^{-1} for ^{13}CO are sufficiently separated to determine the isotope ratio (McDowell, 1970).

A recent development in this field has been the use of an instrument employing the technique called nondispersive infrared heterodyne ratiometry (Irving, et al, 1986). This method can be used to quantitate the CO_2 of a gas mixture. Such a spectrometer measures the intensity of a

heterodyne signal generated from the partial absorption of amplitude-modulated infrared radiation by a pressure-modulated gas sample. This method does not require monochromatic infrared radiation but does require that the intensity of the radiation transmitted through the sample be selectively modulated at the absorption wavelengths of the gas of interest. This process is accomplished by transmitting radiation through a sample of the gas while the density of the gas is modulated in a sinusoidal manner. As yet instruments of this type are still at the prototype stage and could not be used in the proposed studies.

4.1.4 Other techniques

Although not as widely used, the possibility of using Raman scattering for measuring isotope ratios has been investigated. It has been demonstrated that isotopic analyses of nitrogen ($^{14}N^{15}N$) and oxygen ($^{16}O^{18}O$) can be made using an argon laser (Bloom, Harney and Milanovich, 1976).

Activation analysis is another technique that has found some applications. In these methods the desired stable isotope is made to undergo a suitable nuclear transition. The parent isotope is determined by measurement of the resulting radioactive nuclide. For example, the ^{18}O content of water samples as small as 1.5 ul has been determined by charge particle activation (Wood et al., 1975).

Methods for the direct analysis of the deuterium content of aqueous fluids or of organic molecules (via oxidative combustion) include gravimetric analysis and the falling drop method (Schloerb et al., 1951).

None of these other rechniques satisfy the criteria described in the introduction and therefore were not used.

4.1.5 Mass spectrometry

The use of stable isotope tracers in man has not been as widespread as predicted by pioneers in the field. The reluctance to use stable isotopes or to change from radioisotope methodology (brought about by ethical considerations) is due to the laborious sample preparation techniques, unavailability of suitably labelled compounds, high cost of conventional instrumentation and limitations in terms of sample size and precision of such apparatus. This is compounded in the clinical field, where stable isotopes are used to study protein, carbohydrate and fat metabolism, as ^{13}C and ^{15}N studies typically generate large numbers of samples. At present it is necessary to have access to both Isotope Ratio Mass Spectrometry and Gas Chromatography-Mass Spectrometry to carry out precursor and end-product tracer analysis. The reason for needing both types of instrumentation lies in the sample size and precision of isotope ratio analysis required by protein synthesis studies (Preston and McMillan, 1988; Figure 4.3).

The earliest mass spectrometers were developed by Nier and others in the late 1930's and throughout the 1940's (Nier, 1946). They were designed to measure isotopes of the main elements of organic matter, namely, hydrogen, carbon, nitrogen and oxygen. The result of such work was a dual-inlet (for comparison of sample and reference gases), low resolution (analysis of simple gases), double-collector magnetic sector (simultaneous measurement of major and minor isotope) instrument designed for precise heavy isotope measurement of low molecular weight permanent gases (e.g. CO_2 and N_2) which retain their molecular identity under electron bombardment. An Isotope Ratio Mass Spectrometer is capable of detecting 1 labelled molecule of the minor isotope in the presence of 100,000 unlabelled molecules, in its original form, requires

3-30umole of gas for accurate isotope assay, and thus is ideal where sample size is not limiting but high precision isotopic measurements are essential.

In contrast, the Gas Chromatograph-Mass Spectrometer is a high resolution, high sensitivity instrument. It was during the 1960's that the technique of interfacing a gas chromatograph with a mass spectrometer was perfected. The mass spectrometer could be either a quadrupole or magnetic sector analyser. However, as the quadrupole is capable of switching masses more rapidly and is more amenable to computer control than the magnetic analysers in the same price range, it has been more usual to couple this type of mass analyser with a gas chromatograph. Gas Chromatography-Mass Spectrometry for metabolic studies has been further improved by operation in the Selected Ion Monitoring mode (Klein, Haumann and Eisler, 1972; Watson et al., 1973; Gruenke, Craig and Bier, 1980). Gas Chromatography-Mass Spectrometry combines the ability of the gas chromatograph to resolve individual components in a complex mixture and the ability of the mass spectrometer to generate and quantify ions unique to the compound being measured.

Isotope ratios of carbon and nitrogen can be determined with very good precision (better than 0.1% relative standard deviation) by differential isotopic analysis of pure simple gases using the dual inlet dual collector Isotope Ratio Mass Spectrometer developed by McKinney et al (1950), and with adequate precision (10-0.5%, depending on conditions) by Selected Ion Monitoring-Gas Chromatography-Mass Spectrometry (Sweeley et al., 1966). Isotope Ratio Mass Spectrometry and Selected Ion Monitoring-Gas Chromatography-Mass Spectrometry can be thought of as being complementary, the former providing high precision but requiring relatively large samples, the latter handling smaller samples but with reduced

precision. The Gas Chromatography-Mass Spectrometry technique directly incorporates a process of sample purification and is capable of resolving mixtures of compounds, while conventional Isotope Ratio Mass Spectrometry instrumentation dictates that sample preparation is performed off-line. In practical terms, the integral separation process gives Gas Chromatography-Mass Spectrometry a great advantage in the rate of sample throughput and in the confidence of the results that are supposed to relate to individual pure compounds.

There have been over the last 10-15 years several attempts to modify Gas Chromatography-Mass Spectrometry to improve isotope precision of this technique and concurrent developments have sought to simplify and automate the sample preparation and handling for Isotope Ratio Mass Spectrometry.

One approach reported by Sano et al (1976) was a modification of Gas Chromatography-Mass Spectrometry which allowed precise measurement of the ^{13}C:^{12}C ratio in ^{13}C-labelled metabolites of drugs. This new technique enabled ^{13}C-labelled drugs to be used in preference to deuterated drugs whose metabolites frequently showed a kinetic isotope effect of the deuterium label. Other advantages were: simplified identification of peaks and the number of metabolites by combining gas chromatograph and mass spectrometry traces of the separation. Briefly, a mixture of ^{13}C-labelled metabolites was injected into the gas chromatograph and the effluent converted into CO_2 during the passage through a catalytic combustion unit, the resulting CO_2 was subsequently lead into a single collector mass spectrometer. Ions with m/e 45 ($^{13}CO_2$) and m/e 44 ($^{12}CO_2$) were detected alternately at intervals of 0.5 s under computer control of the accelerating voltage. They were able to make isotopic ratio measurements on 5ng of labelled metabolite although the

precision of this measurement was not given. However, the combination of gas chromatograph and with a combustion oven was not new. In 1960, in order to normalise the detector response with regard to carbon number, the effluent from a gas chromatograph was passed through a combustion oven before entering a thermal conductivity detector (Franc and Wurst, 1960). Also in the same year, the effluent from a gas chromatograph was combusted in order to allow on-line elemental analysis of gas chromatograph peaks, the carbon / hydrogen ratio being determined from the CO_2 : H_2 ratio after water combustion product had been reduced to hydrogen (Cacace, Cippollini and Perez, 1960). In another use, the same arrangement followed by a proportional counter was reported for the measurement of ^{14}C and ^{3}H abundances (James and Piper, 1961; Winkleman and Karmen, 1962; Cacace, Cippollini and Perez, 1963).

Matthews and Hayes (1978) reported on work carried out on carbon and nitrogen isotope ratios using essentially the same scheme as Sano and coworkers but with improved instrumentation and selective traps to remove combustion products other than those of interest. These investigators emphasised the capabilities of the new technique (termed Isotope Ratio Monitoring-Gas Chromatography- Mass Spectrometry) namely, the ability to separate a single compound from a complex mixture, the speed of Simultaneous Ion Monitoring-Gas Chromatography-Mass Spectrometry and the improved isotope ratio measurement of their Gas Chromatography-Mass Spectrometry instrumentation. They claimed that this technique could measure nitrogen and carbon isotope ratios down to natural abundance levels for any organic component (or components) that could be resolved gas chromatographically.

Another approach, to introduce rapid automatic sample preparation for Isotope Ratio Mass Spectrometry was described by Preston and Owens

(1983) who interfaced an automatic elemental analyser on-line to a low resolution, multiple collector Isotope Ratio Mass Spectrometer (termed Continuous Flow-Isotope Ratio Mass Spectrometry). In this technique the combustion furnace is placed before the Gas Chromatograph which is designed to separate simple gases. In addition the rapid semiautomatic analysis of total nitrogen and nitrogen isotope ratio measurements using the single step Dumas combustion of the biological sample is used rather than the more routinely used Kjeldahl digestion to ammonium for total nitrogen analysis and the oxidation of ammonium to nitrogen gas by the Rittenberg technique (Springson and Rittenberg, 1949b) for isotope analysis (San Pietro, 1957). A similar scheme was described by Marshall and Whiteway (1985). The Continuous Flow-Isotope Ratio Mass Spectrometer is not only able to carry out more rapid analysis but because of the much simpler sample preparation in comparison to that of Kjeldahl-Rittenberg, the technique is able to provide a more sensitive, reliable isotope ratio. These workers also suggest that the Isotope Ratio Mass Spectrometer could provide more precise isotope ratio detection for the scheme described above by Matthews and Hayes. Although mass spectrometry had been used before as a detector for combustion analysis (Van Meter, Bailey and Brodie, 1951; Barsdate and Dugdale, 1965) and laboratory built Dumas combustion apparatus had been developed for nitrogen isotope analysis (Fiedler and Proksch, 1972; Wada et al., 1977) the implications for automatic rapid precise carbon and nitrogen isotope analysis in the continuous flow mode had not previously been described. Preston and Owens (1985) reported rapid automatic carbon isotope analysis and total carbon content with the same technique as described above. Continuous Flow-Isotope Ratio Mass Spectrometry has since

undergone further refinement to become a fully automated technique (Barrie et al., 1989).

In summary, it is clear from the discussion above that mass spectrometry is the most suitable technique for the stable isotope analysis of samples from protein synthesis studies in man since it fulfills the criteria described in the introduction. However, it is also clear that, for such studies, Continuous Flow-Isotope Ratio Mass Spectrometry offers an alternative to the conventional methodology which involves both Isotope Ratio Mass Spectrometry and Gas Chromatography-Mass Spectrometry. Therefore, Continuous Flow-Isotope Ratio Mass Spectrometry was the technique used for stable isotope analysis in this work.

4.2 Continuous Flow-Isotope Ratio Mass Spectrometer (Figure 4.1)

The Continuous Flow-Isotope Ratio Mass Spectrometer used in this study was made of two basic modules; (A) A biological sample converter (ROBOPREP-CN, Europa Scientific Ltd., Crewe, UK.) which was interfaced directly to (B) a dual collector Isotope Ratio Mass Spectrometer (MM 602, VG Isogas, Winsford, UK.) by a simple variable leak valve and both modules were controlled by a single computer (C).

(A) The biological sample converter is a software controlled sample preparation system employing the Dumas principle and is specifically designed to use a mass spectrometer as a detector. Samples are introduced by automatic gravity fed autosampler into an oxidation tube in the presence of a pulse of oxygen. The CO_2 and N_2 are purified and separated before being transferred to the mass spectrometer by helium carrier gas. The timing and size of the O_2 pulse are software controlled enabling the O_2 pulse to be optimised to suit the size and type of sample.

For nitrogen only measurements, CO_2 is chemically trapped but when carbon only measurements are made, the software switches the CO_2 trap out of the gas flow. The instrument was fitted with a septum inlet after the oxidation furnace (Figure 4.1) for injecting breath CO_2 samples. The helium gas flow rate was maintained at 60 ml/min. The sample preparation system can be further subdivided into its functional units; (1) Autosampler (2) Oxidation furnace (3) Reduction furnace (4) Gas scrubber tubes (5) GC column.

(1) This is a pneumatically operated sample delivery device with a removable 64 position sample carousel. The sample sits in a well where it is purged with helium at approximately 40 ml/min. When the carousel is activated the sample moves forward in a shaft and drops into the oxidation furnace following a pulse of O_2. The next sample drops into the purge well ready for the next cycle. A window situated on top of the sampler allows visible confirmation of the correct flash combustion conditions.

(2) Many different combustion catalysts have been tested for use in quantitative elemental analysis, and have been reviewed extensively by Ma and Gutterson (1974, 1976, 1978). The catalyst suitable for Continuous Flow-Isotope Ratio Mass Spectrometry must (a) produce quantitative combustion (b) not devitrify silica (c) not decompose under reaction conditions and (d) not retain sample combustion products and thus produce memory effects. The first of these restrictions is imposed because incomplete combustion could be accompanied by isotopic fractionation and more directly because fragment ions arising from residual materials such as hydrocarbons might appear at masses 28, 29, 44, or 45 thus causing serious errors in isotope ratio measurements. The oxidation stage of the preparation system (Figure 4.2) was filled with granular chromium (III) oxide (Elemental Microanalysis Ltd., Okehampton, U.K.) and held at 980°C.

The oxidation stage also contains a layer of silver wool, which removes any halogens.

(3) The products of the oxidation stage are passed to the reduction furnace (Figure 4.3) which contains copper granules at 550 ^{o}C where excess oxygen is absorbed and nitrogen oxides are reduced to elemental nitrogen. Specifically for samples (containing ammonium sulphate) used in this work, the reduction stage has a large capacity to trap sulphur dioxide.

(4) There are two gas scrubbing tubes, one of which removes water and the other removes CO_2. The water scrubbing tube (Figure 8) contains granular anhydrous magnesium perchlorate and trapping of CO_2 in the other tube (Figure 4.2) is achieved by filling the tube with 'carbosorb' followed by a layer of magnesium perchlorate (BDH Ltd., Poole, UK.). The CO_2 trap can be switched out of the gas stream if CO_2 is the gas of interest.

(5) The gas stream passes into the gas chromatograph where the gases of interest are separated and then bled into the mass spectrometer via a variable leak valve. The gas chromatograph column was kept at a temperature of 135^{o}C for nitrogen, carbon and breath CO_2 analysis.

(B) The classical dual inlet dual collector Isotope Ratio Mass Spectrometer is ideally suited for measuring very low enrichments in the easily purified end-products of metabolism, urea and CO_2. The instrument derives its sensitivity for determining very small differences in isotopic enrichment (approximately 0.0001 atom% excess) by (a) by measuring only small pure gas molecules, (b) simultaneously measuring both the major and minor isotope-derived ions with a set of Faraday cup collectors for ion current measurement, optimised for each ion beam (c) always measuring the isotope ratio of the sample gas against the isotope ratio of

an equal pressure of reference gas of known isotopic content via periodic switching with a dual gas inlet system. Although Isotope Ratio Mass Spectrometry has been under continuous evolution since the 1930's, the instrument has been tedious to use. With the recent application of the microcomputer to instrument control systems such instruments can now operate automatically.

At present, the limitation of the Isotope Ratio Mass Spectrometer technique is not the instrument, but the sample preparation. Because Isotope Ratio Mass Spectrometer measures small gas molecules placed in an inlet system of finite volume, determination of isotopic enrichment involves converting umole amounts of compound in pure form to the proper gas. The end-products of metabolism, typically with low levels of enrichment have therefore been the main sample type for isotopic measurement by Isotope Ratio Mass Spectrometer: CO_2 can be readily trapped from expired air in mmole amounts (Schoeller et al., 1977): urea and ammonia can be isolated from urine and converted to N_2 usually via reaction of ammonium salt with lithium hypobromite (Ross and Martin, 1970). In contrast, few circulating metabolic substrates can be routinely isolated in pure form in suitable amounts for Isotope Ratio Mass Spectrometry. For example, the leucine in approximately 30ml of plasma must be isolated from all other carbon containing compounds to yield sufficient CO_2 for isotope analysis by Isotope Ratio Mass Spectrometry.

In this work the Isotope Ratio Mass Spectrometer interfaced to the biological sample converter consists of a converted Nier type analyser that contains an ion source, 90^{o} flight tube and ion collectors all within a vacuum envelope. For convenience and to reduce the volume, the vacuum envelope is crescent-shaped with the source and dual faraday bucket collectors housed at opposite ends. Ionisation is caused by the electron

bombardment of the gas sample in the source region and the required ions so generated are then extracted from the source region by an electrostatic field. The Isotope Ratio Mass Spectrometer source filament material was thoriated iridium, the electron energy 70eV and the trap current 260uA. The ions are subjected to an accelerating voltage and ejected into a fixed magnetic field which separates different masses. The ion current charge (major or minor) imparted to its respective collector bucket, resulting from the ion beam impact, is converted by an electrometer amplifier into a measurable voltage.

(C) Computer control of Continuous Flow-Isotope Ratio Mass Spectrometry was performed by a custom software package (Europa Scientific Ltd.) residing in a IBM-PC compatible computer. The software acts via an interface card which has high precision voltage to frequency converters to quantify simultaneously each ion beam current, that is, integrating and computing the isotope ratio, then adding the isotope peaks to give the total elemental content of the simple gas. The interface card also contains a programmable 10volt direct current output to control the mass spectrometer accelerating voltage and switches and sensors which in turn control and monitor the following functions of the sample preparation system; (1) the timing and operation of the autosampler (2) the timing and size of the oxygen pulse (3) the switching in or out of the CO_2 trap from the gas circuit. The software package also carries out data storage and analysis following a run of samples, calculating drift and background values (Barrie et al., 1989).

4.2.1 Sample combustion

The sample combustion containers were aluminium and heated to 450°C for 4h to reduce the carbon and nitrogen blank to a minimum, accepting that the flash combustion process may be less efficient than with tin containers. The analytical sample time was varied according to the samples under analysis. The oxygen pulse size selected for each batch of samples was calculated by adding 2ml to the volume required by the maximum sample size plus that required to oxidise the combustion container. The oxygen pulse was 99.998% pure and the helium 99.9998% pure (Air Products Ltd.). Very pure grades of these gases are needed for the analysis of small nitrogenous samples as this minimises carrier baseline and oxygen blank. The samples analysed in this work produced little ash on combustion compared to the usual sample type that is subjected to combustion analysis. Sample ash build-up in the vertical oxidation stage of the preparation system is undesirable as it can raise the combustion region from the hottest part of the furnace. Also as the ash may contain alkaline and alkaline earth oxides it will cause devitrification if it comes in contact with the quartz combustion tube. To avoid such problems an alumina tube with a plug of silica wool and a layer of chromic oxide was inserted into the quartz combustion tube. The removal of sample ash was accomplished by removing the alumina liner while the furnace was at the 800°C standby setting and replacing it with another (for subsequent deashing). The sample ash was removed after approximately 200 samples when end-products were be analysed and after 70 samples when precursors (submicromolar samples) were analysed. The reason was that the elemental content of the precursor samples was much lower and therefore required minimal peak tailing and lower background levels of carbon and nitrogen. The oxidation stage was replaced after approximately 2000

sample analyses. The reduction stage was also replaced at this time, and regenerated in situ with pure hydrogen after approximately 700 analyses. The scrubbers for water and CO_2 were replaced after approximately 1000 samples.

4.2.2 Carbon isotope analysis by Continuous Flow-Isotope Ratio Mass Spectrometry

The estimation of carbon isotopic enrichment in Continuous Flow-Isotope Ratio Mass Spectrometry is carried out after combustion of organic material to CO_2 when the aluminium boat containing the purified amino acid (in this case leucine) drops into the oxidation stage. The CO_2 is then carried by helium through the copper reduction stage to remove oxygen and through a water scrubber. The CO_2 trap was isolated automatically during the entire analytical cycle. The sample is finally carried through a gas chromatographic column (Carbosieve S, (60-80 mesh, 50cm x 1/4", Phase Separations, UK.) to resolve CO_2 from nitrogen and any trace hydrocarbons. The ^{13}C isotope ratio was calculated from the ratio of integrated peak signals of mass/charge (m/z) 44 ($^{12}C^{16}O_2$) and m/z 45 ($^{13}C^{16}O_2$) from the collector buckets of the mass spectrometer (see 4.2(B)). The m/z 45 also contains a small contribution from $^{12}C^{16}O^{17}O$ which was corrected for (Craig, 1953) in the mass spectrometry software. Atom% ^{13}C was calculated using the formula;

$$100/(R+1)$$

where R is the ratio m/z 44/45 corrected for ^{17}O contribution and scaled to a working ^{13}C standard (L-alanine, Sigma Chemical Co. Ltd, Dorset, UK.). The working standard was calibrated against sucrose of known ^{13}C enrichment. The typical amount of carbon generated from

plasma and intracellular leucine was of the order of 20ug split as a peak over 10 fractions (Figure 4.4). The analytical cycle time was 7.6 min.

In the case of breath CO_2 a needle was fitted to the luer stopcock of the syringe and this was flushed by discarding 10ml of the sample. The remaining 10ml was then injected through a septum inlet of the Continuous Flow-Isotope Ratio Mass Spectrometry preparation system (Figure 4.1) into the helium carrier stream. The breath sample was carried by the helium through the copper reduction stage, removing oxygen, through the water scrubber and through the gas chromatograph column to resolve CO_2 from N_2 and any trace hydrocarbons. It is desirable to resolve N_2 from CO_2 (although there is no isobaric interference) to avoid any baseline disturbance, principally due to the great excess of nitrogen ions generated in the Isotope Ratio Mass Spectrometer ion source. In the mass spectrometer the ion beams are quantified and ^{13}C isotope ratio calculated as described above. The septum inlet was positioned after the oxidation stage to avoid oxidising any trace hydrocarbons to CO_2 and thus not compromise the isotope ratio analysis. Both oxidation and reduction tubes were kept at the standby temperatures (800°C and 400°C respectively) during the breath CO_2 analysis (Figure 4.1). The working standard for the breath CO_2 analysis was a calibrated gas mixture (10% CO_2, 90% N_2, Corning Medical, Essex, UK.). The time from injecting the breath sample to the measurement of both total CO_2 and ^{13}C isotope ratio (analytical cycle time) was 4min.

4.2.3 Nitrogen isotope analysis by Continuous Flow-Isotope Ratio Mass Spectrometry

The estimation of nitrogen isotopic enrichment in Continuous Flow-Isotope Ratio Mass Spectrometry is carried out after combustion to

N_2 when the aluminium boat containing the purified amino acid (in this case glycine) or ammonium drops into the oxidation stage. The N_2 is then carried by helium through the copper reduction stage which reduces nitrogen oxides to N_2. The nitrogen is carried through a water scrubber and a CO_2 trap and finally passes through a gas chromatographic column which resolves nitrogen from any trace hydrocarbons. The ^{15}N isotope ratio was calculated from the ratio of integrated peak signals of m/z 28 ($^{14}N_2$) and m/z 29 ($^{14}N^{15}N$). Atom% ^{15}N was calculated using the formula;

$$100/(2R+1)$$

where R is the ratio m/z 28/29, and scaled to a working ^{15}N standard (L-alanine, Sigma Chemical Co. Ltd, Dorset, UK.). The working standard was calibrated against ammonium sulphate of known ^{15}N enrichment. The typical amount of nitrogen generated from plasma and intracellular glycine was in the order of 12ug split as a peak over 10 fractions (Figure 4.5). The typical amount of nitrogen generated from urinary ammonium was 25-100ug. The analytical cycle time was 6.3min.

4.2.4 Calculation of carbon and nitrogen isotope enrichment of leucine and glycine following amino acid separation

The leucine and glycine peaks were collected in 10 fractions which were individually analysed for their isotopic enrichment. On analysis of the leucine fractions, there was increasing carbon rising to a peak in fraction 5 or 6 and then falling to low levels (Figure 4.4). Similarly, isotopic enrichment rose to a peak and then fell. However, the peak isotopic enrichment did not coincide with that of the peak elemental content, but appeared in the subsequent fraction. This finding indicates isotope fractionation by the cation exchange high performance liquid

chromatography separation and has been reported by other workers (Laragh, Sealey and Klein, 1965; Garlick et al., 1989).

To correct for isotope fractionation two approaches can be used. Firstly, all the fractions containing the label can be pooled and then analysed or secondly, to avoid impurities in the sample collection, the sample is collected in several fractions symmetrically about the peak, analysed and a weighted average of the enrichment/specific activity of fractions carried out (Laragh, Sealey and Klein, 1965). With the amino acid separation techniques used in this thesis (see section 3.4.3) it is not possible, for an individual sample, to rule out some contamination of the glycine or leucine peak with an adjacent peak, because operation of the fraction collector was not synchronised with the eluting peak, due to small retention time variations. Therefore, to avoid this problem and to ensure an accurate isotope ratio measurement was obtained, all fractions before, during and following the eluting peak were analysed and a weighted average was taken to calculate the isotope ratio from the 6 fractions which contained greater than 97% (in the case of nitrogen) and 93% (in the case of carbon) of the tracer (Table 4.4). These 6 fractions used for isotope analysis invariably contained at least 80% of the total elemental content of the 10 fractions analysed. Fractions containing less than 0.7ugN would normally be excluded from the final isotope enrichment calculation (see below). This approach is unlikely to introduce a serious error into the measurement of the isotope ratio (Laragh, Sealey and Klein, 1965; Table 4.4; see section 4.2.5).

In the case of ^{13}C enrichment measurement account had to be taken of the contribution of the aluminium combustion container and mobile phase (see section 3.4.3) residue to the carbon content of each fraction, which together were of the order of 2ug$\pm$0.2ug. In practice, this was

achieved by subtracting the carbon content of the last fraction from all the other fractions before a weighted average of the isotope ratio was calculated. The carbon blank of the pulse of oxygen used as an aid to combustion (see section 4.2.1) was negligible. Finally, since only 1 of the 6 carbon atoms in leucine is labelled the isotope ratio was multiplied by 6 to give the true enrichment. It was assumed that there was no variation in the natural abundance of the 5 unlabelled carbon atoms.

The nitrogen blank from the aluminium combustion container and mobile phase residue was of the order of 0.1 $\pm$ 0.04ugN, being much less than that from the oxygen pulse (typically 1.41 $\pm$ 0.04ugN). Samples of below 0.7ugN (i.e., those with a signal-to-background ratio less than 0.5:1) gave poor isotope ratio analysis precision and were excluded from analysis. The typical sample signal-to-background ratio was between 0.5:1 and 5:1, which accounts for the Gaussian shape of the isotope ratio versus fraction number plot (Figure 4.5; see also Matthews and Hayes, 1978).

4.2.5 Sample recovery; precision and accuracy of isotope analysis

As detailed in Chapter 3, sample recovery was approximately 70% for the whole procedure from sample preparation to amino acid separation, with approximately 50% of the original sample being used for isotope analysis.

Precision of isotope ratio analysis improves with increasing sample size. In this work, urinary ammonium analysis was performed at a signal-to-background ratio of approximately 50:1 (25-100ug atom NH^4-N, see section 3.4.4) and breath CO_2 at greater than 100:1 (approximately 4umol CO_2, see section 4.2.2). Analytical precision (coefficient of variation) for these large sample quantities was 0.16% and 0.22%, in natural abundance and low enrichment ^{15}N samples and 0.09% at natural

abundance and 0.11% in low enrichment breath CO_2 samples (Preston and McMillan, 1988).

Analytical precision for amino acids was determined in the free amino acid samples and protein hydrolysates. Repeated analysis was performed on plasma of known glycine concentration which had a known amount of [^{15}N]glycine added. The precision was determined, for the whole procedure, to be 2.77% (see Table 4.1). Similarly, for [^{13}C]leucine the precision of the procedure was determined to be 4.12% (see Table 4.1). The precision of the whole procedure for isotope ratio measurement of low enrichment [^{15N}]glycine (approximately 0.01atom% excess) and [^{13}C]leucine (approximately 0.02atom% excess) of duplicate hydrolysate samples (see section 3.4.2) was calculated. The relative standard deviation as a percentage (coefficient of variation) was 3.97% (n=6) and 5.26% (n=5) for glycine and leucine respectively. The coefficient of variation was calculated from the formula:

Coefficient of Variation = 100xSQRT((SUM(2x(a-b)/(a+b))^2)/2n)

where a and b are the two samples from the same treated tissue, n is the number of duplicates, SQRT is square root and SUM is the sum of the terms (Forsberg et al., 1991).

The accuracy of the whole procedure (sample preparation to amino acid separation) was determined by repeated analysis of plasma which had a known amount of [^{15}N]glycine added to plasma of known glycine concentration. The target value for free [^{15}N]glycine enrichment was 1.00atom%. The accuracy was determined, for the whole procedure, to be 100.34% (see Table 4.1). Similarly, for [^{13}C]leucine (target value 2.59atom%), the accuracy of the procedure was determined to be 103.61% (see Table 4.1).

The accuracy of the Continuous Flow-Isotope Ratio Mass Spectrometry instrumentation used in this thesis has been verified in a International Atomic Energy Authority intercalibration study (Parr and Clements, 1991). Five standards, in a variety of chemical forms, were analysed as described in section 4.2.3. The mean (standard deviation) ^{15}N enrichment of each standard (analysed 6 times) was 0.38125(0.00088), 0.38471(0.00027), 0.45615(0.00110), 0.50223(0.00073) and 2.04665(0.01335)atom% which compared with the mean values determined by 28 laboratories worldwide of 0.38088, 0.38363, 0.45586, 0.50373 and 2.0500atom% respectively. Therefore, analytical precision was better than 0.001 atom% ^{15}N at natural abundance and all results agreed to within 0.005 atom% of the study mean.

As calculations reported in this study are based on the change of enrichment with time, that is, after subtraction of a baseline enrichment from a measured enrichment, accuracy could potentially be compromised by uncertainty in the baseline enrichment of the compound of interest. End-product (urinary ammonium and breath CO_2) and plasma precursor enrichment calculation used the measured (pre-tracer) natural abundance to overcome this problem, however, this was not possible for biopsy samples. Baseline enrichments for tissue protein-bound and free amino acids were assumed to be the same as pre-tracer plasma amino acid enrichment. Uncertainty can be minimised be ensuring sufficient tracer incorporation into the product (optimising tracer dose and protocol duration).

The change in ^{15}N enrichment in liver glycine (approximately 0.02atom% ^{15}N, (see Table 5.12) and muscle glycine (approximately 0.009atom% ^{15}N, see Table 5.12) were above these levels (possibly 0.0005atom% ^{15}N). Similarly, natural abundance variations in ^{13}C (of the

order of 0.0008atom% ^{13}C; Schoeller et al., 1977) are below the measured change in liver protein-bound leucine enrichment (approximately 0.02atom% ^{13}C, see Table 5.13). In practice, the error that natural abundance variations could introduce into measured rates of protein synthesis is likely to be less significant than other uncertainties in the system, such as accurate estimate of tracer enrichment at the site of protein synthesis (see section 2.4, 7.4)

4.3 Study protocol validation

As discussed in Chapter 2 the simultaneous measurement of whole body and tissue protein synthesis can be achieved using a continuous infusion of a labelled amino acid. Furthermore, from the discussion of the relative merits of the two approaches (precursor and end-product methods) it was concluded that a comparison between [^{13}C]leucine and [^{15}N]glycine was required to assess which approach would be best suited for such simultaneous measurements in this work.

To date, [^{15}N]lysine and [^{13}C]leucine have been used to make these measurements in man (Halliday and McKeran, 1975; Rennie et al., 1982a). In contrast to [^{15}N]lysine there are a number of studies which have used a primed continuous intravenous infusion of [^{13}C]leucine to make such simultaneous protein synthesis measurements in man (Matthews et al., 1980; Rennie et al., 1982a; Nair, Halliday and Griggs, 1988). Therefore, it would appear that the precursor approach using [^{13}C]leucine is suitable for simultaneous whole body and tissue protein synthesis measurements. However, with this approach it is necessary to determine the recovery of $^{13}CO_2$, using the CO_2 collection apparatus and isotope analysis methods of the particular laboratory, to correct expired $^{13}CO_2$ enrichment data to give the oxidation rate of [^{13}C]leucine (see section 2.2). This was carried

out in a single subject (fasted overnight for 12h) using a continuous infusion of $NaH^{13}CO_2$ (started at 09:30am) and the percentage recovery of $^{13}CO_2$ calculated for the closed canopy and isotope analysis systems used in this work (see Table 4.5, section 3.6 and 4.2). The recovery of labelled CO_2 was shown to be, on average, 80.6% which is in accord with other reports (Wolfe, 1984b).

From the literature there appears to be no reports, in man, of simultaneous measurement of whole body and tissue protein synthesis using [^{15}N]glycine. This may be due, in the case of [^{15}N]glycine, to the further complication that the metabolic end-product (urea or ammonia) must also be at plateau in order to calculate the whole body protein turnover value.

The use of urinary urea as the end-product of [^{15}N]glycine metabolism (urea being quantitatively the most important end-product of nitrogen metabolism) has resulted in very long infusion protocols (40-60h, due to the large size and relatively slow turnover of the body urea pool) being required before [^{15}N]urea reaches plateau enrichment (Steffee et al., 1976; Winterer et al., 1980). These protocols have been shortened to 18-24h by the use of a priming dose of the tracer in conjunction with the constant infusion (Sim et al., 1980; Jeevanandam et al., 1985). In describing the tracer priming dose relative to the infusion dose rate, Jeevanandam and coworkers (1985) have termed this the 'prime to infusion ratio', although strictly speaking this is not a ratio since it has units of time. However, the term is used in this thesis for convenience. It has been demonstrated using a continuous intravenous infusion of [^{15}N]glycine that a large prime to infusion ratio (prime (mg/kg)/infusion (mg/kg/min)) of approximately 1500min is required to achieve plateau isotopic enrichment in urinary urea within 24h (Jeevanandam et al., 1985).

Such a large prime to infusion ratio would compromise the plateau isotopic enrichment in the plasma and tissue precursor pool and therefore the linear incorporation of labelled amino acid into the tissue required for accurate calculation of the protein fractional synthetic rate. However, the ammonia pool in man is of the order of 150 times smaller than that of urea and therefore will reach an isotopic enrichment plateau more rapidly than urea at the lower prime to infusion ratio required for tissue synthetic rate measurements. Furthermore, it is clear from the literature and work carried out in this laboratory that the isotopic enrichment of urinary ammonia gives similar relative values in both in normal subjects and cancer patients (Fearon et al., 1988) and therefore can be used as the metabolic end-product in the calculation of whole body protein turnover (Garlick and Fern, 1985).

The measurement of protein synthesis in tissues, without the use of complicated formulae and assumptions about the kinetics of [^{15}N]glycine (Stein et al., 1976), requires that the plateau isotopic enrichment in the free amino acid precursor pool is attained rapidly and maintained throughout the duration of the infusion. Stein and coworkers (1976) used a continuous infusion (without a prime dose) of [^{15}N]glycine and demonstrated that plateau isotopic enrichment was reached in the plasma amino nitrogen in approximately 6h, urinary hippurate (reflecting hepatic intracellular [^{15}N]glycine enrichment) in approximately 3h, and urinary ammonia in approximately 4h. Therefore, in order to calculate protein fractional synthetic rates they used formulae (involving a further assumption about the rate of isotopic equilibration in the body's free amino acid pool) based on the work of Garlick and coworkers (1973). In order to shorten the time to plateau isotopic enrichment in the plasma glycine pool it has been calculated that the prime to infusion ratio should be 80min

(Jeevanandam et al., 1985). Recent studies using [^{15}N]glycine to measure protein fractional synthetic rates of hepatic export proteins have used prime to infusion ratio of approximately 30min (Cryer et al., 1986 ; Thompson et al., 1989). These studies have confirmed that prime to infusion ratio of approximately 30min does result in a more rapid attainment of plateau [^{15}N] enrichment in free plasma glycine and urinary hippurate (95% of plateau isotopic enrichment within approximately 30min and 2h of the start of the infusion respectively). Indeed, this is what would be expected from the continuous infusion experiments on rats (using [^{14}C]glycine) which demonstrated a rapid equilibrium between free plasma and liver glycine pools (Fern and Garlick, 1974). Therefore, it is likely that with the prime to infusion dose of approximately 30min that the plateau enrichment of [^{15}N]glycine in the true precursor pool for protein synthesis (probably glycyl-tRNA) is achieved within 3h and is maintained for the duration of the infusion.

From the above discussion it is clear that what is not known is the time to plateau isotopic enrichment in urinary ammonia at the lower prime to infusion ratio of approximately 30min. Therefore, a preliminary study was carried out in 4 normal subjects to establish the time to plateau isotopic enrichment in urinary ammonia and confirm that of free plasma [^{15}N]glycine following a primed continuous infusion of [^{15}]glycine (prime to infusion ratio 30min).

Following a 12h fast a spot urine sample was collected for baseline measurement of urinary ammonia and a venous blood sample was taken for free plasma [^{15}]glycine enrichment. A primed (0.014mg/kg) constant 24h intravenous infusion (0.029mg/kg/h), prime to infusion ratio 30min, of [^{15}N]glycine (99atom%, Tracer Technologies Inc., MA, USA.) was then commenced. All voided urine was collected over the next 24h with timed

collections of 0-3h, 3-6h, 6-12h and 12-24h. The ^{15}N enrichment in urinary ammonia was measured in all urine samples as described in Chapters 3 and 4. The results (Table 4.2) demonstrate that the plateau isotopic enrichment of [^{15}N]ammonia is attained rapidly (probably within 3h) and maintained for the duration of the infusion. There was no significant difference in the [^{15}N]ammonia enrichment over the collection periods 3-6h, 6-12h and 12-24h (analysis of variance, see 4.4). In two subjects the rise to plateau enrichment of free plasma [^{15}N]glycine was followed (Table 4.3). The time to 90% plateau enrichment was within 3h and was maintained for 24h, which is in accord with other published work (Cryer et al., 1986 ; Thompson et al., 1989).

These normal subjects did not undergo surgery and therefore sequential tissue samples were not taken to validate the plateau enrichment of intracellular free glycine. However, at this prime to infusion ratio the plateau ^{15}N enrichment in urinary hippurate (reflecting liver intracellular [^{15}]glycine enrichment) has been reported to be reached in approximately 2h (Cryer et al.,1986; Thompson et al., 1989). Therefore, it is probable that isotopic equilibrium in the liver occurs before this since there will be a delay in the excretion of hippurate into the urine. With reference to the continuous infusion of glycine in man there appears to be no information in the literature which allows us to determine the time taken to reach isotopic equilibrium in muscle. From work carried out in animals the time taken to reach a plateau of free [^{15}N]glycine enrichment in muscle is considerably longer, due to its slow turnover rate and large pool size (Waterlow, Garlick and Millward, 1978i). However, even a 4 fold increase in the time taken to isotopic equilibrium in the muscle compared with liver (from the above discussion, approximately 2h for liver) would probably introduce no more than a 20% overestimation (using a 20h continuous

infusion) in the muscle protein fractional synthetic rate, calculated simply, as described above. The duration of the continuous infusion of [^{15}N]glycine was determined not only by the aim to make protein synthetic measurements in the whole body, liver and muscle, but also the practicability of carrying out a continuous overnight infusion in hospitalised subjects. In previous work from this laboratory an 18h continuous infusion of [^{15}N]glycine, using ammonia as an end-product, has been shown to be of similar discriminatory power as the urea end-product in the measurement of whole body protein turnover in normal and weight-losing cancer patients (Fearon, 1986; Fearon et al., 1988). Therefore, a primed continuous infusion of [^{15}N]glycine of approximately 20h duration was used.

In summary, given that the protocol in the clinical studies (Chapters 5 and 6) was to last 20 hours and that intrahepatic glycine would achieve isotopic equilibrium before urinary hippurate and urinary ammonia, any uncertainty in estimating the integrated precursor pool enrichment in the liver and presumably the muscle would introduce only a small error into the calculated fractional synthetic rate. From the above study, and the work of Jeevanandam and coworkers (1985), the simultaneous measurement of whole body and tissue protein synthetic rates in man using an intravenous infusion of [^{15}N]glycine requires that the prime to infusion ratio should be between 30 and 80min and that ammonia should be used as the metabolic end-product. Therefore, the prime to infusion ratio of [^{15}N]glycine chosen for the studies described in Chapters 5 and 6 was 60min.

4.4 Statistical Methods

The statistical software used to carry out all comparisons of data was Minitab, release 7 (Minitab Inc., PA, USA.). All data is presented as individual values (except where the volume of data precluded such treatment) with the mean and standard deviation of the values to facilitate comparison with other work. Repeat measurements over time were assessed for statistical significance using the Kruskal Wallis test (analysis of variance). Paired and unpaired data from the studies presented in this thesis were tested for statistical significance using the Wilcoxon signed rank test and the Mann-whitney test respectively (Wallenstein, Zucker and Fleiss, 1980). Correlations between paired values was tested using simple regression statistics.

Table 4.1.

Precision and accuracy of 13C and 15N isotope ratio analysis of plasma free leucine and glycine

The precision and accuracy of the method was determined by repeated separation and analysis of plasma in which the free [^{15}N]glycine enrichment was brought to 1.00 atom% by precise volumetric additions.

Mean isotope ratio analysis (n=4)	1.0034 atom%	^{15}N
Standard deviation from the mean	0.0277 atom%	^{15}N
Precision (CV)	2.77 %	
Accuracy*	100.34 %	

The precision and accuracy of the method was determined by repeated separation and analysis of plasma in which the free [^{13}C]leucine enrichment was brought to 2.50 atom% by precise volumetric additions.

Mean isotope ratio analysis (n=4)	2.5902 atom%	^{13}C
Standard deviation from the mean	0.1068 atom%	^{13}C
Precision (CV)	4.12 %	
Accuracy*	103.61 %	

* Accuracy was calculated according to the formula:

(1+(M measured isotope ratio-T isotope ratio)/T isotope ratio))x100

CV, coefficient of variation = (Standard deviation/M)X100

Where M is mean and T is true

Table 4.2

Urinary [15N]ammonia kinetics during a primed constant infusion of [15N]glycine

Time		0-3h	3-6h	6-12h	12-24h
		Urinary [^{15}N]ammonia (atom% excess)			
Subject	A	0.0307	0.0385	0.0401	0.0412
	B	0.0304	0.0401	0.0390	0.0412
	C	0.0385	0.0427	0.0423	0.0449
	D	0.0375	0.0415	0.0414	0.0423
Mean		0.0343	0.0407	0.0407	0.0424
S.D.		0.0043	0.0018	0.0014	0.0017

Measurement of urinary [^{15}N]ammonia (atom% excess) during a primed (0.014mg/kg) constant 24h intravenous infusion (0.029mg/kg/h) of [^{15}N]glycine in timed urine collections.

Table 4.3

Plasma glycine kinetics during a primed constant infusion of [15N]glycine

Time (h)	Subject E (% mean plateau enrichment)	Subject A (% mean plateau enrichment)
0.33	59	
0.66	58	
1.5	74	
2.0	72	58
3.0	106	88
4.0	90	113
5.0	124	
6.0	121	
8.0	110	90
10.0	85	
12.0		114
16.0		117
24.0		78

Measurement of plasma glycine kinetics during a primed (0.014mg/kg) constant 24h intravenous infusion (0.029mg/kg/h) of [^{15}N]glycine in two normal subjects. Values expressed as a percentage of the mean plateau enrichment.

ble 4.4

lculation of [15N]glycine and [13C]leucine isotope enrichment.

ycine analysis

mple 1

raction N,	ugN	APE	ngN15 E	C-14(dpm)
1	0.447	0.0081	0.036207	272
2	0.693	0.0412	0.285516	340
3	1.981	0.0234	0.463554	833
4	2.444	0.3497	8.546668	2397
5	3.304	0.5656	18.68742	4437
6	2.366	0.6569	15.54225	5151
7	1.691	0.5954	10.06821	3774
8	1.113	0.2641	2.939433	2057
9	0.803	0.0534	0.428802	1156
10	0.531	0.0194	0.103014	527
tal	15.373		57.10109	20944
fract.	12.899	0.436061	56.24755	18649
Total	83.90685		98.50521	89.04221

mple 3

raction N,	ugN	APE	ngN15 E	C-14(dpm)
1	0.417	0.0042	0.017514	226
2	0.761	0.0121	0.092081	370
3	1.89	0.0253	0.47817	913
4	2.32	0.3188	7.39616	2627
5	2.989	0.5482	16.3857	4859
6	2.138	0.6368	13.61478	5628
7	1.532	0.5871	8.994372	4131
8	0.658	0.2634	1.733172	2262
9	0.452	0.0502	0.226904	1273
10	0.294	0.0111	0.032634	352
tal	13.451		48.97149	22641
fract.	11.527	0.421639	48.60236	20420
Total	85.69623		99.24623	90.19036

Sample 2

Fraction N,	ugN	APE	ngN15 E	C-14(dpm)
1	0.821	0.0094	0.077174	251
2	1.147	0.0478	0.548266	792
3	1.745	0.2364	4.12518	2436
4	2.442	0.4337	10.59095	3668
5	3.411	0.592	20.19312	5530
6	2.523	0.6277	15.83687	5852
7	2.045	0.5689	11.63401	3696
8	1.153	0.1182	1.362846	1917
9	0.742	0.0239	0.177338	874
10	0.478	0.0086	0.041108	328
Total	16.507		64.58686	25344
6 fract.	13.319	0.478587	63.74298	23099
% Total	80.68698		98.69341	91.14189

Sample 4

Fraction N,	ugN	APE	ngN15 E	C-14(dpm)
1	0.656	0.0325	0.2132	204
2	1.875	0.0734	1.37625	561
3	2.311	0.2859	6.607149	1717
4	2.419	0.5706	13.80281	4403
5	3.431	0.7001	24.02043	5508
6	1.717	0.6469	11.10727	3621
7	1.815	0.3739	6.786285	1819
8	0.861	0.0777	0.668997	1037
9	0.37	0.0233	0.08621	323
10	0.232	0.0193	0.044776	217
Total	15.687		64.71339	19410
6 fract.	12.554	0.501776	62.99295	18105
% Total	80.02805		97.34145	93.27666

ucine analysis

mple 5

raction C,	ugC	APE	ngC13 E	C-14(dpm)
1	0.878	0.0085	0.07463	178
2	1.847	0.0126	0.232722	645
3	2.704	0.0413	1.116752	950
4	3.501	0.1701	5.955201	1275
5	4.29	0.1905	8.17245	2618
6	3.892	0.2823	10.98712	2996
7	3.751	0.2443	9.163693	2532
8	3.172	0.0901	2.857972	1773
9	2.132	0.0249	0.530868	1195
10	1.105	0.0185	0.204425	220
tal	27.272		39.29583	14382
fract.	21.31	0.179508	38.25318	12144
Total	78.13875		97.34668	84.43888

mple 7

raction C,	ugC	APE	ngC13 E	C-14(dpm)
1	0.914	0.0078	0.071292	184
2	1.533	0.0117	0.179361	698
3	2.399	0.0373	0.894827	1266
4	3.107	0.1521	4.725747	2622
5	3.685	0.1704	6.27924	3084
6	3.466	0.257	8.90762	2606
7	3.23	0.2422	7.82306	1924
8	2.756	0.0986	2.717416	1287
9	1.852	0.0282	0.522264	736
10	0.97	0.0111	0.10767	193
Total	23.912		32.2285	14600
fract.	18.643	0.168148	31.34791	12789
Total	77.96504		97.26768	87.59589

Sample 6

Fraction C,	ugC	APE	ngC13 E	C-14(dpm)
1	0.821	0.0091	0.074711	193
2	1.732	0.0134	0.232088	682
3	2.521	0.0405	1.021005	990
4	3.228	0.1673	5.400444	1321
5	3.989	0.1871	7.463419	2703
6	3.386	0.2452	8.302472	2499
7	3.264	0.2122	6.926208	2112
8	2.76	0.0783	2.16108	1479
9	1.855	0.0231	0.428505	1003
10	1.009	0.0194	0.195746	229
Total	24.565		32.20568	13211
6 fract.	19.148	0.163331	31.27463	11104
% Total	77.9483		97.10905	84.05117

Sample 8

Fraction C,	ugC	APE	ngC13 E	C-14(dpm)
1	0.863	0.0077	0.066451	228
2	1.822	0.0114	0.207708	913
3	2.682	0.0349	0.305748	1353
4	3.432	0.1446	1.197768	1942
5	4.732	0.1625	6.842472	3996
6	3.051	0.2676	4.957875	3691
7	2.841	0.2316	7.602516	3115
8	2.402	0.0856	5.563032	2031
9	1.614	0.0254	1.381584	1171
10	0.774	0.0191	0.196596	268
Total	24.213		28.32175	18708
6 fract.	19.14	0.138294	26.46941	16128
% Total	79.04845		93.45966	86.20911

nalysis of individual plasma samples for glycine enrichment (15N) and leucine enrichment (13C) as described in section 4.2.4. ugN ug nitrogen, APE atom% excess, ngN15 E ngN15 excess, C-14(dpm) C-14(disintegrations per minute), 6 fract. total for 6 fractions, % Total percentage of Total value.

Table 4.5

The recovery of expired 13CO2

Time (min)	Total CO_2 (ml/min)	Total CO_2 (mmol/min)	$^{13}CO_2$ (AP)	$^{13}CO_2$ (APE)	$H^{13}CO_2$ Infused (APE.mmol/min)	$H^{13}CO_2$ recovered (%)
0			1.11327			
79	218	9.732	1.14567	0.0324	0.4444	71.0
88	218	9.732	1.14993	0.03666	0.4444	80.3
96	218	9.732	1.14983	0.03656	0.4444	80.1
220	214	9.554	1.15426	0.04099	0.4444	88.1
231	214	9.554	1.15215	0.03888	0.4444	83.6

The recovery of expired $^{13}CO_2$ in a single subject (fasted for 12h) using a primed (0.38mmol) continuous infusion (4.54umol/min) of $NaH^{13}CO_3$ (99atom%, Tracer Technologies Inc. MA, USA).

An average of 80.6% of the labelled bicarbonate was recovered as expired $^{13}CO_2$.

It was calculated as follows;

((Total CO_2 (mmol/min)x $^{13}CO_2$ (APE))/ $H^{13}CO_2$ Infused (APE.mmol/min))x100

AP atom%, APE atom% excess

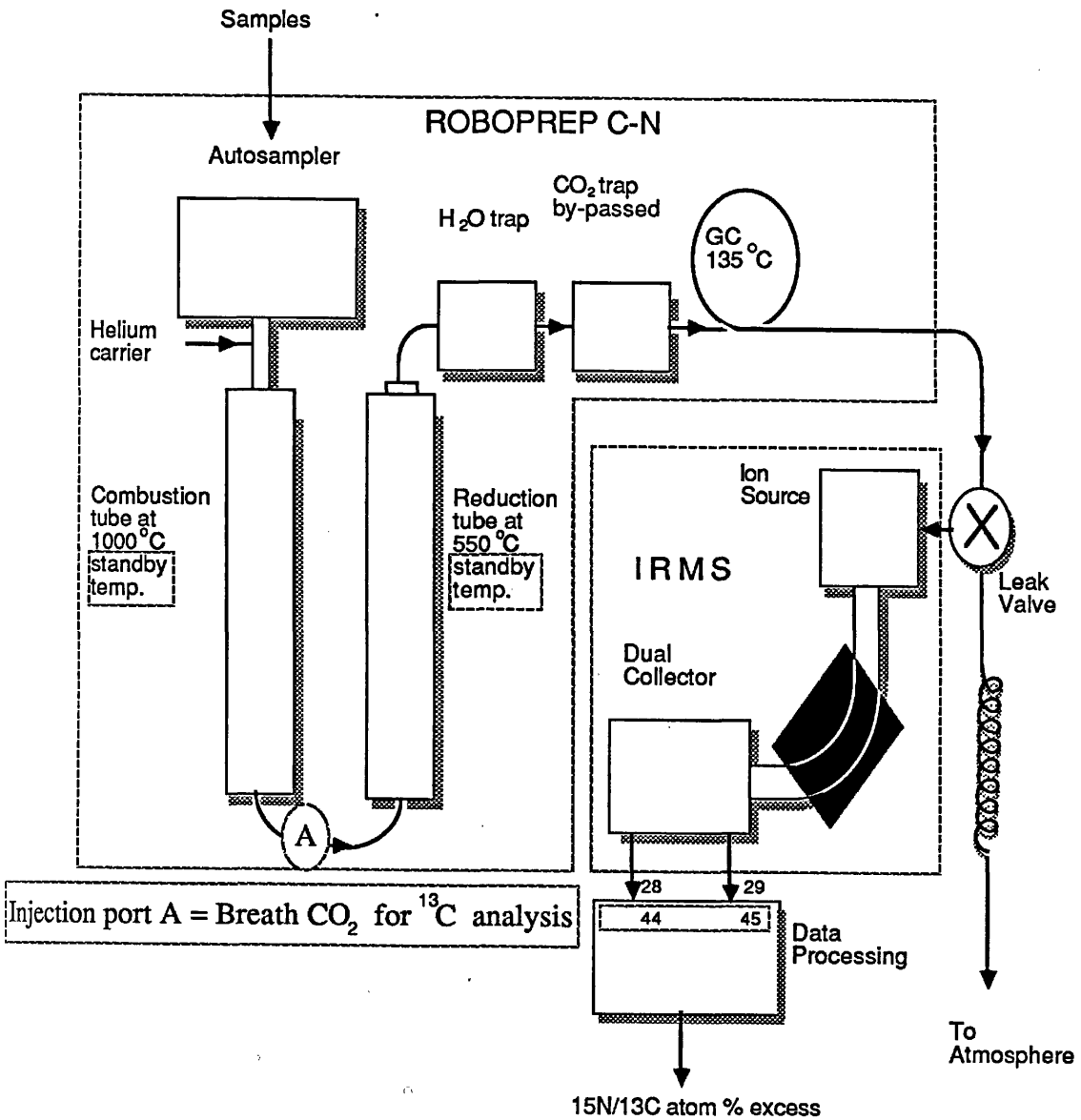


Figure 4.1 15N and 13C analysis by Continous Flow-Isotope Ratio Mass Spectrometry

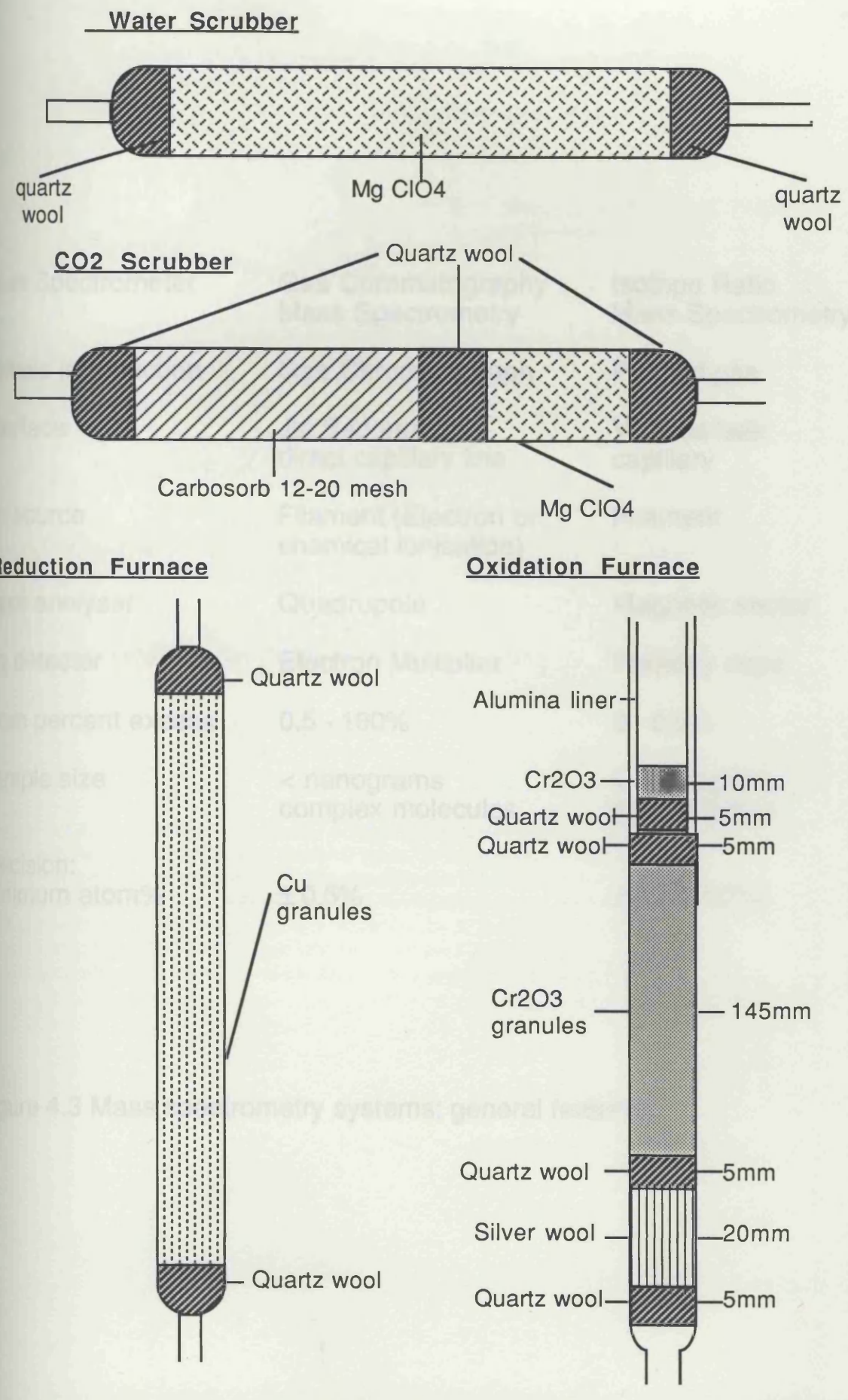


Figure 4.2 Tube packings for Continous Flow-Isotope Ratio Mass Spectrometer

Mass Spectrometer	Gas Chromatography Mass Spectrometry	Isotope Ratio Mass Spectrometry
Sample introduction	Gas Chromatograph	Purified gas
Interface	Jet Separator or direct capillary line	Viscous leak capillary
Ion source	Filament (Electron or chemical ionisation)	Filament
Mass analyser	Quadrupole	Magnetic sector
Ion detector	Electron Multiplier	Faraday cups
Atom percent excess	0.5 - 100%	0 - 0.5%
Sample size	< nanograms complex molecules	> micrograms simple gases
Precision: minimum atom%	± 0.5%	± 0.00007%

Figure 4.3 Mass spectrometry systems: general features

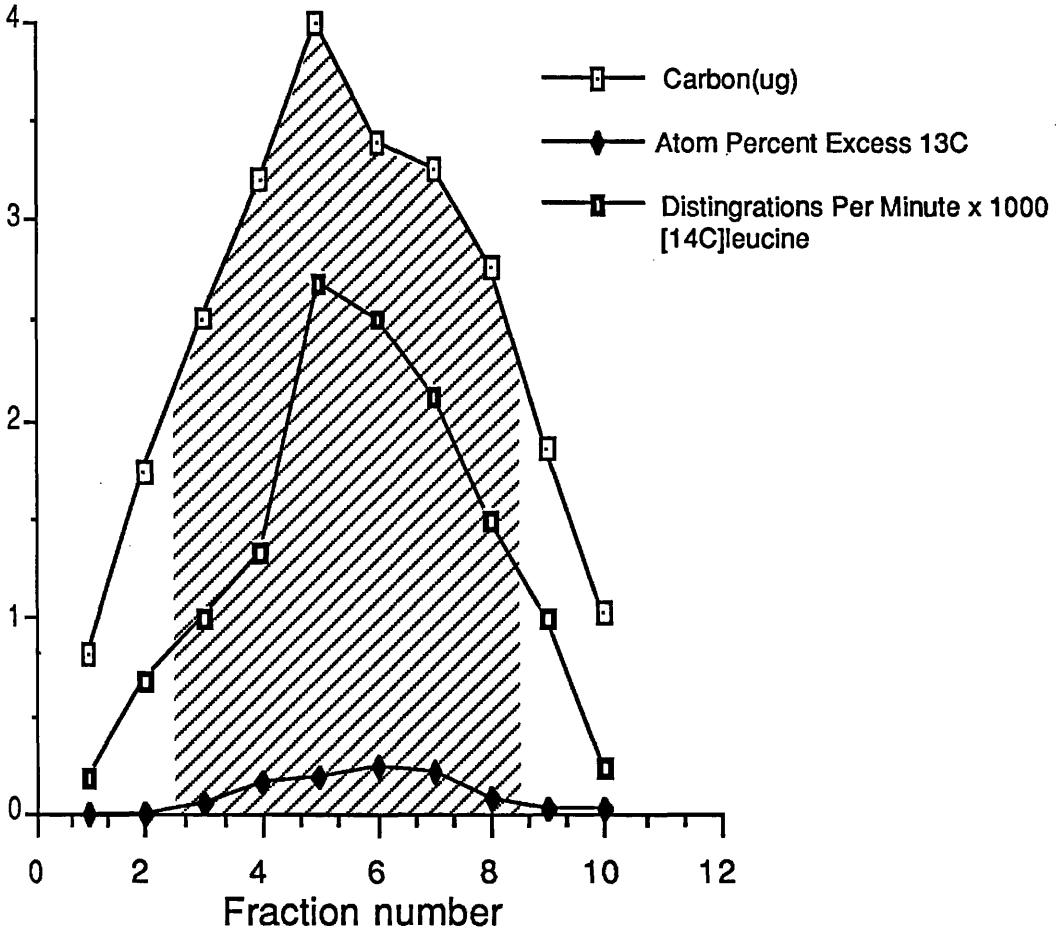


Figure 4.4 Analysis of the leucine peak eluted from cation-exchange high performance liquid chromatography separation of amino acids. The shaded area represents the fractions taken for the weighted average calculation of the isotopic enrichment of [13C]leucine (carbon blank subtracted from each fraction), that is, the weighted average isotopic enrichment from the 6 fractions (multiplied by 6, to account for only 1 of the 6 carbon atoms in [13C]leucine being labelled). The area under the peak used in the calculation was more than 80% of the total carbon measured (confirmed by [14C]leucine).

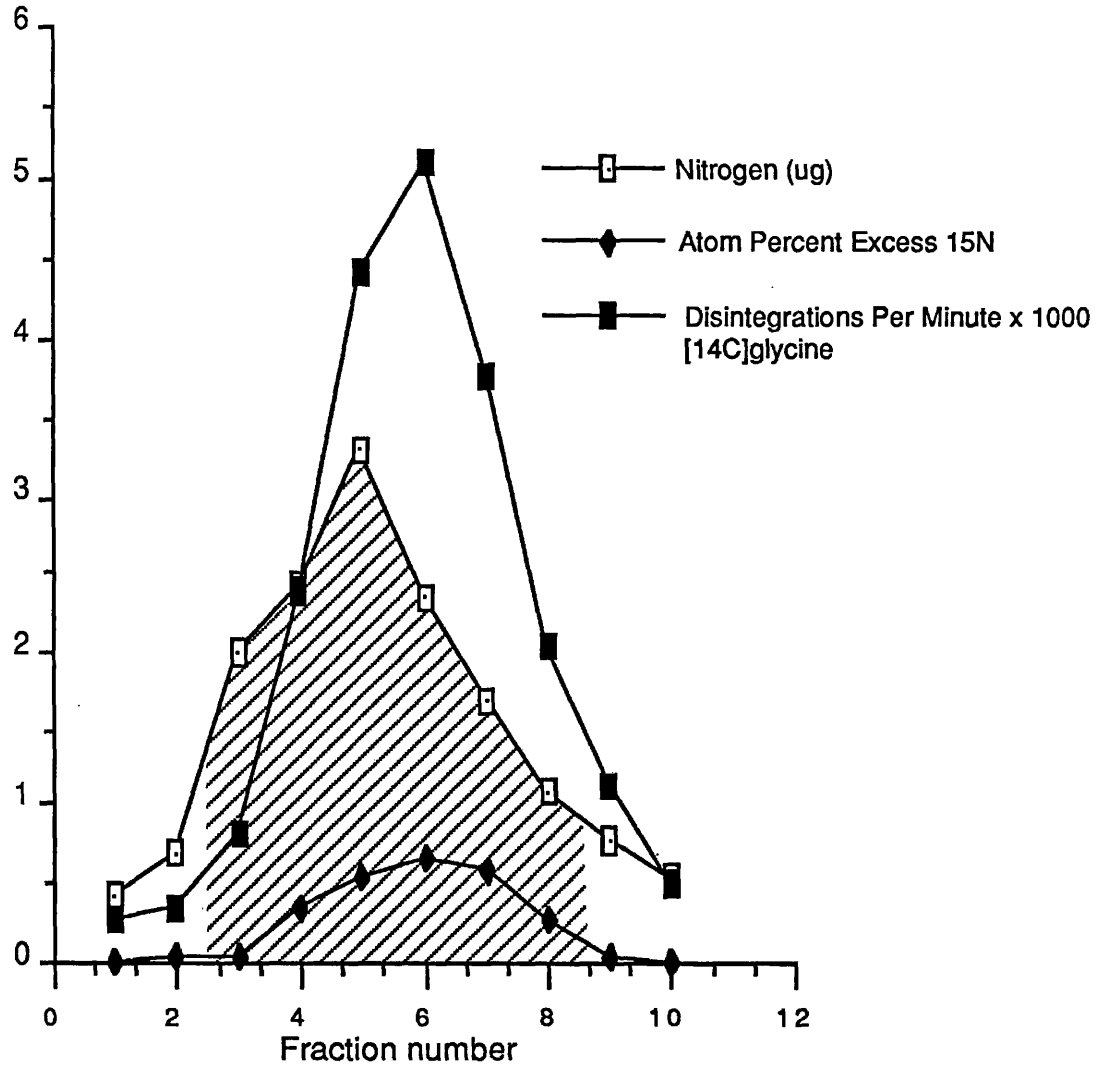


Figure 4.5 Analysis of the glycine peak eluted from cation-exchange high performance liquid chromatography separation of amino acids.The shaded area represents the fractions taken for the weighted average calculation of the isotopic enrichment of [15N]glycine, that is , the weighted average isotopic enrichment from the 6 fractions. The area under the peak used in the calculation was more than 80% of the total nitrogen measured in the peak (confirmed by [14C]glycine).

CHAPTER 5: THE SIMULTANEOUS MEASUREMENT OF WHOLE BODY, FIXED LIVER AND MUSCLE PROTEIN SYNTHETIC RATES IN NORMAL SUBJECTS USING [15N]GLYCINE AND [13C]LEUCINE

5.1 Introduction

As discussed in Chapter 2, in order to measure simultaneously whole body, liver and muscle protein synthetic rates a primed constant infusion protocol can be used. However, there is still the question as to which amino acid would be the most representative tracer in the clinical situation? Most recently [^{15}N]glycine or [^{13}C]leucine have been used in human studies. Leucine has been used more frequently in fractional synthesis measurements in man, particularly with reference to muscle. This has followed the move away from the use of [^{15}N]glycine to measure whole body protein synthesis due to the uncertainty over which end-product to measure and the many metabolic routes that the nitrogen label can take. Nevertheless, both amino acids appear to have similar discriminatory power when applied various disease states and starvation (Garlick and Fern, 1985).

One of the major differences between glycine and leucine based methodologies is that the latter is a branched chain essential amino and an isotope of carbon is used to label the amino acid. Whereas, glycine is a non-essential amino acid and a nitrogen label is used. Therefore, with reference to liver, where the branched chain amino acids (in contrast to glycine and the other amino acids) pass through the liver without their blood concentration being altered significantly, the protein synthetic rates measured by these amino acids could be different. Finally, the use of these amino acids to measure whole body protein turnover requires different protocols: leucine has a small metabolic pool and rapid turnover whereas

glycine has a large metabolic pool and slower turnover rate. The result of this is that to measure whole body protein synthesis, depending on the size of the priming dose, a leucine protocol takes 4-12h and the glycine protocol 12-24h.

In deciding which tracer to use for the present study the following criteria were considered to be important.

(a) that the tracer give valid fractional synthetic rates using the analytical approach described in Chapters 3 and 4.

(b) that the tracer give valid fractional synthetic rates when the tissue samples are taken during an operative procedure.

With reference to (a) an important consideration in our analytical approach is the amount of sample required for mass spectrometric analysis. Although the Continuous Flow-Isotope Ratio Mass Spectrometer was optimised for small sample analysis approximately 1umole of the amino acid was required for reliable analysis. The concentration of free amino acids is generally higher in the intracellular compartment and the ratio of tissue to plasma concentration varies considerably for each amino acid. In the rat the tissue to plasma ratio for leucine is 2.1 in liver and 0.6 in muscle whereas, for glycine it is 10.1 in liver and 13.6 in muscle (Lunn, Whitehead and Baker, 1976). In man there appears to be no published values for liver but, in muscle the ratio for leucine is 1.2 and for glycine is 6.5 (Bergstrom et al., 1974). Therefore, depending on the size of the biopsy the amount of free leucine in both liver and skeletal muscle will be much less than glycine and may be below the amount required for isotopic analysis by Continuous Flow-Isotope Ratio Mass Spectrometry.

With reference to criteria (b), when biopsies are removed in an operative protocol, consideration must be given to the effect of anaesthesia on the measurement of fractional synthetic rates. There is

evidence that anaesthesia alters protein synthesis in different tissues (Heys et al.,1989; Ferguson et al., 1989). Furthermore, it has been suggested that pre-medication alters the concentration and enrichment of plasma leucine (and presumably intracellular leucine) (Rennie and MacLennan, 1985). Therefore, as the fractional synthetic rate is calculated from the precursor amino acid enrichment and the enrichment of the amino acid incorporated into tissue protein then alteration of either of these parameters by anaesthetic work-up will result in an inaccurate estimate of the fractional synthetic rate.

The aim of the study reported in this Chapter was to establish, in otherwise healthy individuals, which amino acid would best estimate whole body, liver and muscle protein synthetic rates with biopsies taken at the time of elective surgery. The more reliable method would then be used to examine the basis of increased whole body protein turnover in cancer cachexia.

5.2 Materials and methods

5.2.1 Subjects

Six patients with cholelithiasis whose last attack of cholecystitis had been at least three months prior to surgery, who had normal biochemical liver function tests and no history of jaundice were studied. The patients had been admitted for elective cholecystectomy and were weight stable. They were weighed (by myself and another investigator) wearing light night attire on beam balance standing scales in the morning of the day that the [^{15}N]glycine infusion was commenced (Weylux 424, UK.). The balance used was accurate to 0.1kg and therefore when the weight of the individual was expressed to the nearest kg no error in the measurement was apparent.

These subjects were selected since they were healthy individuals in all respects except that they had gallstones and required a laparotomy at which incidental muscle and liver biopsies could be obtained. They had no evidence of an acute phase response and were judged clinically to be free of other metabolic or endocrine disorders. None were pyrexial, had clinical or radiological evidence of infection, were receiving steroids, or severely anaemic. All patients had a normal serum urea and creatinine. The study was approved by the local ethical committee. All patients were informed of the purpose and procedure of the study and all gave written consent.

5.2.2 Experimental design (Figure 5.1)

Following a twelve hour fast, a urine sample was collected for baseline enrichment measurement of urinary ammonia. A catheter was inserted into an antecubital vein of each arm. One catheter was used for the infusion of [^{15}N]glycine and the other for sampling blood. Prior to the

start of the infusion, 10ml of blood (heparinised) was taken for basal ^{15}N enrichment of free glycine and ^{13}C enrichment of free leucine and blood analyses. A further 10ml of blood was taken and divided between EDTA and plain glass containers (see analyses Chapter 3). The infusate and drip set were connected to the subject via a volume infusion pump (IVAC Corp., CA., U.S.A.) which had been calibrated over two 24h periods. A primed (4.2mg) constant 20h intravenous infusion (100mg/24h) of [^{15}N]glycine (99atom%, Tracer Technologies Inc. MA, USA.) was then commenced at 18:00 on the day before the operation. All voided urine was collected for the next 18h, providing estimates of nitrogen, creatinine and 3-methylhistidine excretion. In the present study the urinary nitrogen collection was over the last 18h of a 30h fasting period. Therefore, the effect of the lag in reduced urinary nitrogen excretion, postabsorptively, would be unlikely to cause a serious error in the nitrogen excretion estimate (Munro, 1964) and so correction, for changes in the plasma urea concentration over this period, of this estimate was not applied (Fern et al., 1981). A spot urine sample was taken at 18h to determine ^{15}N enrichment of ammonia at plateau. After 15h of the glycine infusion the leucine protocol was started. This consisted of a bolus of $NaH^{13}CO_3$ (57mg) and [^{13}C]leucine (65mg) followed by a constant intravenous infusion of [^{13}C]leucine (65mg/h) over approximately 6h. Further plasma samples were taken just before the start of the leucine infusion, at 120 and 60min prior to surgery and at the time of surgery after induction of anaesthesia. These samples were used to establish the plateau enrichment of free [^{15}N]glycine and [^{13}C]leucine and their concentration in plasma before and during anaesthetic work up. The plasma was separated by centrifugation at 1500g for 10min at 4°C and promptly frozen at -30°C. Within 10min of the start of the operation (14:00) a wedge liver biopsy

was taken together with a biopsy of rectus abdominus muscle (wet weight 215-859mg, Table 5.3a) and rapidly frozen at -80°C. From these biopsies isotopic enrichment of the labelled amino acids in the free amino acids and protein hydrolysate was measured. In addition, the total RNA and free amino acid concentrations of the liver and muscle biopsies was measured.

5.2.3 Analytical Methods

Reagents; All reagents used were analytical grade and water was doubly distilled and deionised (18Mohm).

Isotopic measurements; The study protocol involved the measurement of ^{15}N and ^{13}C in three different sample matrices: urine (ammonia) or breath (CO_2), plasma and liver or muscle tissue. Sample preparation and tracer analysis was as described in sections 3.4 and 4.2.

Resting energy expenditure; Resting energy expenditure and respiratory quotient were measured using an indirect calorimeter with a rigid canopy (Kinney et al., 1964) as described in section 3.6.5. Each resting energy expenditure study began at 09:30h on the day of the operation. Prior to each study, patients remained in bed from the time of wakening.

Tissue R.N.A. analysis; R.N.A. was analysed by U.V. absorption of the supernatant following extraction from tissue (dry weight 20mg) with perchloric acid (Chapter 3).

Analysis of plasma albumin and intracellular amino acid concentrations, together with urinary nitrogen, creatinine and 3-methylhistine concentration were carried out as described in Chapter 3.

5.2.4 Calculations

As discussed in Chapter 2 (2.2, 2.3) whole body protein turnover can be derived from the whole body amino acid flux measurements by using a simplified model of protein metabolism (Figure 2.1). In this model, the labelled amino acid enters a single free amino acid pool from which it can either be oxidised, giving rise to labelled CO_2 or urinary nitrogen (e) or it can be incorporated into protein. In this study whole body amino acid flux has been measured by a continuous infusion of [^{13}C]leucine and [^{15}N]glycine under assumed steady state conditions. Whole body protein turnover from such measurements involves different calculations for each labelled amino acid.

From section 2.2, using [^{13}C]leucine, amino acid flux can be calculated from the expression

$$Q = i\,[E_i/E_p - 1]$$

Where i is the infusion rate (umol/kg/h), E_i is the enrichment of the [^{13}C]leucine infused (atom% excess) and E_p is the [^{13}C]leucine enrichment in plasma at isotopic equilibrium (atom% excess). The rate of $^{13}CO_2$ released by leucine oxidation (umol ^{13}C/kg/h),

$$e = [FCO_2 \times ECO_2/W] \times [60 \times 41.6/100 \times 0.81]$$

Where FCO_2 is the CO_2 production (ml/min), ECO_2 is the $^{13}CO_2$ enrichment in expired air at isotopic steady state (atom% excess) and W, the subject's weight (kg). The constants 60min/h and 41.6umol/ml (at standard temperature and pressure) convert FCO_2 to umol/h, the factor 100 changes atom% excess from a percent to a fraction and the factor 0.81 accounts for the fraction of $^{13}CO_2$ released by [^{13}C]leucine oxidation but not released from the body bicarbonate pool into expired air (see section 4.3, Table 4.5, Wolfe, 1984b). The rate of leucine oxidation is given by

$$E = e/[E_p - E_i] \times 100$$

from which the rate of leucine incorporation into protein can be calculated

$$S = Q - E$$

Both estimate of rates of leucine flux (Q) and rates of leucine incorporation into protein (S) are expressed as umol/lg/h. The values can be converted to give estimates of whole body protein turnover and whole body protein synthesis in gProtein/kg/d by multiplying the leucine values by the constant [Y/24]/590 where the term Y/24 normalises the infusion time Yh to 1 day and the term 590 converts umol leucine to gram protein. The 590umol leucine/g protein factor is from Matthews and coworkers (1980) who derived it by averaging values for leucine content of protein in human flesh and other mammalian muscles. Clearly, the leucine content of whole-body protein (a heterogenous group of proteins with different turnover rates) can only be an estimate and therefore values for whole body protein turnover calculated from values for leucine turnover must also be estimates.

From section 2.3 using [^{15}N]glycine, the total nitrogen turnover (flux) can be calculated from the expression

$$Q = d / S_{max}$$

Where d is the infusion rate (mg^{15}N/kg/h), S_{max} is the ^{15}N enrichment in urinary ammonia at isotopic equilibrium (atom% excess) and Q is mgN/kg/h. Total nitrogen synthesis can be calculated

$$S = Q - E$$

Where E is the nitrogen excretion (gN) over the period of the infusion. The values can be converted to give estimates of whole body protein turnover and whole body protein synthesis in gProtein/kg/d by multiplying the nitrogen values by the constant [Y/24]x6.25 where the term Y/24 normalises the infusion time Yh to 1 day and the term 6.25 converts gram nitrogen to gram protein (Picou and Taylor-Roberts, 1969). Faecal and

insensible nitrogen losses were not measured or corrected for since the error likely to be introduced into the calculation of whole body protein synthesis, in a fasting protocol, would be less than 5% (Cheng et al., 1978; Calloway, Odell and Margen, 1971), and is less than the variability of the method (Preston, 1987).

In order to calculate tissue protein synthesis the uptake of the tracer from the plasma or intracellular fluid into liver and muscle protein is measured. The rate of tissue protein synthesis was calculated from the simple formula;

$$K_s = P(_t)x100/A$$

Where K_s is the fractional rate of protein synthesis (%/day), $P(_t)$ is the enrichment of labelled amino acid in liver or muscle protein (corrected for the natural background enrichment in the plasma free amino acid) at the end of the incorporation period (atom% excess) and A is the area under the curve for precursor enrichment (atom% excess x infusion time of the labelled amino acid). As stated earlier (see 4.3) the measurement of protein fractional synthetic rates using the above formula assumes that the plateau isotopic enrichment in the free amino acid precursor pool is attained rapidly and maintained throughout the duration of the infusion of the labelled amino acid. It would appear from the literature and work presented in this thesis (see 4.3) that this approach is not in serious error.

The liver fractional synthetic rate was calculated using either the ^{15}N enrichment of plasma free glycine to represent the precursor pool or the ^{15}N enrichment of free glycine from the liver homogenate. The liver fractional synthetic rate was calculated using the ^{13}C enrichment of plasma free leucine to represent the precursor pool as there was insufficient sample to measure the isotopic enrichment of free leucine from the homogenate. The rate of hepatic protein synthesis in grams per day

was calculated assuming that liver mass is 2% of body weight and that 146g/kg of liver is protein (Geigy Scientific Tables). The muscle fractional synthetic rate was calculated using either the ^{15}N enrichment of plasma free glycine to represent the precursor pool or the ^{15}N enrichment of free glycine from the muscle homogenate. The rate of muscle protein synthesis in grams per day was calculated assuming that muscle mass is 29.3% of body weight and that 172g/kg of muscle is protein (ICRP, 1975).

Data is presented as the mean value and standard deviation.

5.3 Results

The clinical features of the subjects are shown in Table 5.1. The subjects were all female, weight stable and had albumin concentrations in the normal range (36-44g/l). The body mass index (Weight/(Height^2)) of subjects is shown in Table 5.1.

Individual rates of whole body protein turnover, whole body protein synthesis, urinary nitrogen excretion and resting energy expenditure are presented in Table 5.2. The mean rates of whole body protein turnover and synthesis measured using [^{15}N]glycine were 3.5 and 3.1gP/kg/d respectively. In contrast, the mean rates of whole body protein turnover and synthesis measured using [^{13}C]leucine were 3.8 and 3.5gP/kg/d respectively. Mean urinary nitrogen excretion was 4.2gN/d and resting energy expenditure 1757kcals/d.

Tissue protein synthesis rates are shown in Tables 5.3 and 5.4. The sample from subject 4 was lost during sample preparation (Table 5.4). It must be emphasised, with reference to the liver, that in the prolonged constant infusion protocol the rates of synthesis measured are predominantly of fixed hepatic proteins. This is due to labelled amino acid which is incorporated into synthesised liver export proteins being rapidly

removed from the liver. When the isotopic enrichment of plasma free glycine was taken to represent the enrichment in the precursor pool (for protein synthesis) the mean values for the liver and muscle protein fractional synthetic rates were 8.1 and 2.3%/d respectively. In contrast, when the isotopic enrichments of free glycine derived from the liver and muscle homogenates, were taken to represent the precursor pool enrichment these rates increased to 10.9 and 2.8%/d. The mean rates of liver and muscle protein synthesis expressed in grams of protein were calculated to be 21.9 and 96.4gP/kg/d respectively. These rates were calculated using the enrichment of free glycine in the tissue homogenate and assumed that liver weight was 2% of total body weight and that 146g in each kg of liver was protein (Geigy Scientific Tables). Similarly, it was assumed, in this group of females, that muscle weight was 29.3% of total body weight and that 172g in each kg of muscle was protein (ICRP 23, 1975). When fixed liver and muscle protein synthesis was calculated as a proportion of whole body protein synthesis the mean values were found to be 11.3 and 50.1% respectively.

Insufficient sample was available to measure the isotopic enrichment of free leucine derived from the homogenate and because of this the measurement of [^{13}C]leucine incorporation in the muscle biopsies was not carried out. However, if the isotopic enrichment of plasma free leucine is taken to represent the enrichment in the precursor pool for protein synthesis the mean value for the liver protein fractional synthetic rate was 7.1%/d (Table 5.5). The mean rate of liver protein synthesis expressed in grams of protein was calculated to be 13.9gP/kg/d. This was calculated using the same assumptions as described above for tissue mass and protein content. When liver protein synthesis was calculated as a proportion of whole body protein synthesis the mean value was found to

be 5.9%. The isotope enrichment data, from which whole body and tissue protein fractional synthetic rates were calculated, for [^{15}N]glycine and [^{13}C]leucine are presented in Tables 5.12 and 5.13 respectively.

The enrichment of [^{15}N]glycine and [^{13}C]leucine in plasma was measured in samples taken 120, 60 and 0min before the start of surgery (Table 5.6). [^{15}N]glycine and [^{13}C]leucine enrichment measurements at these time-points were not significantly different.

The mean value for liver RNA concentration was 4.2ugRNA/mg protein and for muscle 0.5ugRNA/mg protein (Table 5.10).

The plasma amino acid concentrations just before the start of the leucine infusion and at the time of induction of anaesthesia are shown in Table 5.7. There was a significant increase in leucine ($p<0.05$) concentration although there were no significant changes in the total or essential amino acids concentrations.

The free amino acid profile of the liver and skeletal muscle biopsies are shown in Table 5.8. The mean total amino acid concentrations were 210 and 117nmol/mg wet weight respectively.

Urinary creatinine and 3-Methylhistidine excretion are shown in Table 5.9. The mean creatinine excretion in the 18hr urine collection was 4.5mmol/l. The mean excretion of 3-Methylhistidine was 32umol/mmol creatinine.

5.4 Discussion

In this study whole body protein turnover and synthesis values using [^{15}N]glycine or [^{13}C]leucine were similar to those obtained in previous work from this laboratory (Fearon et al., 1988) and other published work (Chapter 7, Table 2). The use of an ammonia end-product alone underestimates whole body protein turnover compared to urea and the end-product average (Fern and Garlick, 1985a). However, the ammonia end-product has been shown to have similar discriminatory power as urea in comparative studies (Fearon et al., 1988; see section 7.2.2, Table 7.2). The whole body turnover and synthesis values measured by [^{15}N]glycine and [^{13}C]leucine did not rank with each other as reported by other workers (Golden and Waterlow, 1977). Nor did they rank with urinary nitrogen or resting energy expenditure. Furthermore, there was no significant correlation (section 4.4) between urinary nitrogen excretion and measured resting energy expenditure although such a positive relationship has been suggested by Kinney (1988). All the subjects were over-weight as defined by the body mass index (Table 5.1, Owen, 1988).

One potential criticism of the use of a prolonged infusion of a labelled amino acid is that, because it can be incorporated into and released from protein over the time of the infusion, there may be recycling of the label and this could result in significant errors in the flux measurement. Schwenk and coworkers (1985) reported that when a [^{13}C]leucine infusion was given to normal subjects for 4h and 24h this resulted in a 25% underestimate of the leucine flux in the 24h compared to the 4h infusion. They attributed the difference in the flux measurement to the recycling of label. There was, however, no evidence of recycling over the 4h infusion and they concluded that it occurred after this time. Therefore, with respect to the duration of [^{13}C]leucine infusion in this

study (approximately 5h) it is likely that there was no significant recycling of the label. In the present study the [^{15}N]glycine infusion lasted approximately 20h and therefore it is possible that significant recycling may have occurred. However, the shorter infusion duration of [^{13}C]leucine compared to that of [^{15}N]glycine would not necessarily result in proportionately less recycling since the free leucine and end-product pool sizes are some 5 fold smaller (see section 5.1) and therefore respond more rapidly to label re-entry. Furthermore, using a 20h infusion of [^{15}N]glycine, there was little evidence of significant recycling, which would be demonstrated by a continuing positive gradient in the urinary ammonia or plasma glycine enrichment data (Tables 4.2, 4.3, 5.6), or indeed gross underestimation of rates of whole body protein turnover (see discussion above). Lastly, the prime to infusion ratio chosen (see 4.3) for the continuous infusion of [^{15}N]glycine is unlikely to result in the precursor pool enrichment rising substantially above the final plateau isotopic enrichment in tissues given the data in the literature (Cryer et al., 1986; Thompson et al., 1989) and Tables 4.2, 4.3. Therefore, it would appear that the duration of the primed continuous infusions of [^{13}C]leucine and [^{15}N]glycine used in this study were not associated with significant label recycling.

It has been reported, using a continuous infusion of [^{13}C]leucine, that premedication and the induction of anaesthesia results in acute changes in the enrichment of plasma leucine (Rennie and Maclennan, 1985). These changes are not consistent with a steady state and may therefore invalidate calculation of whole body and fractional protein synthetic rate measurements using models which assume steady state kinetics. In order to establish whether such changes in plasma [^{15}N]glycine and [^{13}C]leucine enrichment were evident in the current protocol, plasma

samples were taken 120 and 60min before, and also at the time of biopsy. The mean values for the 6 patients at each time point is given in Table 5.6. There was no significant difference (analysis of variance, see 4.4) in the plasma glycine or leucine enrichment between the time points leading up to the operation. This suggests that with glycine and leucine a stable plateau of isotopic enrichment was maintained and thus steady state kinetics could be used to calculate results.

Unfortunately, there was insufficient free leucine in the tissue homogenate for isotopic analysis by the analytical methods used. Nevertheless, when plasma leucine enrichment was used to calculate the liver protein fractional synthetic rate, the values obtained ranked approximately to those when plasma glycine enrichment was used (Tables 5.3, 5.5). Therefore, with the protocol and methodology used in the present study and if it can be assumed that the plasma free amino acid enrichment is a valid estimate of the true precursor enrichment (for protein synthesis, see section 2.4), it would appear that either tracer is suitable for the measurement of whole body and tissue protein fractional synthetic rates. However, in this work, the precision and accuracy of [^{13}C]leucine enrichment measurement was poorer than that of [^{15}N]glycine (see section 4.2.5). Furthermore, the plasma [^{13}C]leucine enrichment measurements were more variable than those of [^{15}N]glycine (Table 5.13, 5.12). With reference to the criteria in the introduction (see section 5.1) it would appear that [^{15}N]glycine best estimates whole body, liver and skeletal muscle protein fractional synthetic rates. Therefore, [^{15}N]glycine was chosen for the study of protein synthetic rates in weight-losing cancer patients described in Chapter 6.

It has been suggested that the enrichment of free glycine in tissue homogenate estimates better the true precursor enrichment for protein

synthesis than that in plasma (Fern and Garlick, 1974). The large concentration gradient between intracellular free glycine and plasma free glycine concentrations (see Tables 5.7, 5.8) may also be indicative of the likely source of glycine to charge t-RNA. The fractional synthetic rate for hepatic fixed protein was found to be 10.8%/d (Table 5.3) when estimated from homogenate free glycine enrichment. This was calculated to represent the synthesis of approximately 22g of protein per day and would therefore account for 11.3% of protein synthesis in the whole body. Comparative data from other studies is very scarce. In one of the few reported studies in humans, Stein and coworkers determined the fixed hepatic protein fractional synthetic rate to be approximately 15%/d (Stein et al, 1978a). However, the latter study was on cancer patients in which the incorporation of ^{15}N into the alpha amino nitrogen of liver protein was measured and therefore the results are not directly comparable since the hepatic free alpha amino pool has a very different composition to that of the protein bound pool. Data on fixed hepatic protein synthesis in animals is also limited but it has been reported that in pigs (approximately 75kg), fixed liver protein synthesis is 23.3%/d, accounting for 10.0% of the whole body protein synthesis rate (Garlick, Burk and Swick, 1976).

Fractional synthetic rates for total liver protein synthesis in growing lambs, rats and piglets have been reported to be between 70-115%/d, accounting for 12-15% of whole body protein synthesis (Attaix et al., 1988). In comparison with values for adults such higher fractional synthetic rates reflect the fact that these animals are generally of lower body weight and are growing. In contrast, there is no published data on total liver protein synthesis in man. However, there is data on the major liver export protein, albumin, which accounts for approximately 70% of the protein secreted by the liver in normal man (Fleck, Colley and Myers,

1985). Indeed, a similar protocol to that described in this work has been used to measure the fractional synthetic rates of liver export proteins via the incorporation of [^{15}N]glycine into plasma albumin and fibrinogen (Gersovitz et al., 1980; Thompson et al., 1989). The albumin synthetic rate has been reported to be 4-6%/d of whole body protein synthesis (Gersovitz et al., 1980). Using these results and the data in Table 5.3 total liver synthesis would account for 17-20%/d in normal man. It would appear, therefore, that hepatic protein synthesis contributes substantially to the rate of protein synthesis in the whole body and may thus account for a significant proportion of whole body protein synthesis observed in disease states.

Measurement of skeletal muscle protein synthesis in human studies has been mainly carried out using [^{13}C]leucine with sampling from the vastus laterlis muscle. Therefore, some caution must be taken in comparing values in the literature with those obtained in the present study (using [^{15}N]glycine and sampling from the rectus abdominus muscle). What is clear is that the values obtained using [^{15}N]glycine show greater variation and are higher than those obtained by other workers using [^{13}C]leucine (Table 5.11) but are similar to those obtained by Halliday and McKeran (1975) using [^{15}N]lysine (sampling vastus laterlis muscle). However, the values for muscle protein synthetic rates given in Table 5.11 have assumed either the plasma or intracellular precursor isotopic enrichment to be the best estimate of the true precursor (see section 2.4) and therefore it is not surprising that the measured synthetic rates are quite different. The effect of using the plasma or intracellular amino acid enrichment for the calculation of the muscle protein fractional synthetic rate is considered further in section 7.4.

It has been demonstrated in animal studies that biopsies from different muscle sites have different synthetic rates. For example, in the fully grown rat the fractional synthetic rate of the soleus and diaphragm muscles is approximately double that in the gastrocnemius and plantaris muscles (Waterlow, Garlick and Millward, 1978e). This is thought to be due to the fact that certain muscles such as rectus abdominus are aerobic in type whereas others such as vastus lateralis are anaerobic. Furthermore, different amino acids are likely to give the different values for muscle protein synthesis (Waterlow, Garlick and Millward, 1978f), presumably due to differences in their metabolism and the amounts in the tissue (for further discussion see section 7.4). Halliday and McKeran (1975) estimated that muscle protein synthesis accounted for approximately 53% of whole body protein synthesis in fasted normal subjects. However, using [^{13}C]leucine Rennie and coworkers, (1982a) estimated this value to be 43%. These values are similar to those in the present study where skeletal muscle protein synthesis was estimated to be 50% of whole body protein synthesis. In contrast, Young and Munro (1978) estimated indirectly that muscle protein synthesis probably accounts for about 30% of whole body protein turnover.

It is clear from the literature that interpretation of blood or tissue amino acid profiles must be carried out with a view to the method and conditions of sampling. In the present study the tissue biopsies were taken at the start of surgery (within 10 minutes of skin incision) and the plasma samples taken at the induction of anaesthesia. Therefore, it is not surprising that the amino acid profiles that were obtained from the tissues in this study are different from those obtained in studies involving non-operative biopsy procedures e.g., needle biopsy. In the baseline plasma samples (Table 5.7) the most significant difference in the amino

acid profile was the low glutamine concentration (when compared to the normal range, Table 5.7). This may be characteristic of this group of patients since most of the other amino acids in the profile are depressed. Alternatively, it could be due to stress or the postabsorbtive state. Studies in the rat in the postabsorbtive state suggest that glutamine is extracted by the gut as a source of nitrogen and used for the synthesis of alanine which is then released by these tissues (Matsutka et al., 1973). The amino acid profile of the pre-operative plasma samples (Table 5.7) taken just prior to surgery was not significantly different from that of the baseline samples with the exception of the leucine concentration which was increased significantly ($p<0.05$). This confirms the observation of Rennie and Maclennan, (1985) who attributed an increased plasma leucine concentration to the effect of premedication and/or anaesthesia. Alternatively, the increase in plasma leucine concentration may be due to the on-going leucine infusion. Interpretation of the tissue free amino acid profiles is difficult, as there are few literature values for man obtained under similar conditions (especially liver).

The free glutamine concentrations of the liver biopsies obtained in the present study were very low whereas, glutamate and histidine concentrations were very high when compared to the normal rat liver (Herbert, Coulson and Hernandez, 1966). It has been shown in rats that liver protein synthesis is reduced rapidly on the induction of anaesthesia by up to 37%. Furthermore, there is some evidence that premedication has a similar effect (Heys et al., 1989). However, it is not known what effect premedication/anaesthesia has on the free amino acid profile of the rat/human liver. It may be that the general prominence of glutamate reflects increased transamination in the liver at this time.

The amino acid profiles of the skeletal muscle biopsies (rectus abdominus) in the present study are very similar to those of muscle taken by needle biopsy, except that alanine is markedly increased (Wernerman et al, 1985). However, a similar pattern has been reported by Wernerman et al, (1985) after a 1h infusion of stress hormones. The lack of alteration in the amino acid profile as a whole is in accord with the small effect of premedication/anaesthesia on skeletal muscle protein synthesis (Heys et al., 1989).

The variability of the amount of RNA per unit protein is much greater than previously reported (Millward et al., 1973; Jepson and Millward, 1989). The method has been used by a number of workers and gave reasonable precision with a normal liver biopsy (see section 3.5.3). Furthermore, the study samples were carefully treated prior to RNA analysis. Therefore, it is not clear what the source of variability was in the method used. However, it is clear with such variability of the data, interpretation must be limited.

The resting expenditure measurements and their variability observed in the normal subjects, in the present study, was similar to that reported previously for other groups of normal individuals (Fearon et al., 1988; Melville et al., 1990). The liver and skeletal muscle together probably account for the major part of protein turnover in the whole body (Waterlow, Garlick and Millward, 1978b) (approximately 60% for fixed liver and skeletal muscle protein synthesis in this study). Furthermore, the minimum proportion of energy expenditure associated with protein synthesis is thought to be approximately 15-25% of the total body heat production (Reeds, Wahle and Haggerty, 1982). In various disease states protein synthesis rates in these compartments may differ widely, the balance being reflected in whole body protein synthesis. It has been

reported that the rate of whole body protein turnover derived from labelled amino acid kinetics is increased in cancer (Jeevanandam et al., 1984; Fearon et al., 1988). However, the magnitude of the change observed in such studies without an accompanying increase in energy expenditure has led some authors to question the nature of the relationship between tracer amino acid kinetics and actual whole body protein turnover (Clague et al., 1982; Fearon et al., 1988). Therefore, measurement of whole body and tissue synthetic rates in weight-losing cancer patients might allow some resolution of these apparently contradictory findings. The results of such a study are reported in the following Chapter (6).

Table 5.1

Characteristics of patients studied as 'healthy' individuals

		Age (yrs)	Sex	Weight (kg)	Height (cm)	BMI	Albumin (g/l)
Subject	1	70	F	74	157	30	40
	2	53	F	69	157	28	40
	3	74	F	60	152	26	39
	4	67	F	70	152	30	43
	5	58	F	58	152	25	41
	6	39	F	86	155	36	41
Mean		60		70	154	29	41
S.D.		13		10	2	4	1

BMI, Body Mass Index=weight(kg)/[height(m)]^2

Table 5.2

Whole body protein kinetics and resting energy expenditure in healthy subjects

	[^{15}N]glycine		[^{13}C]leucine			
	WBPT (gP/kg/d)	WBPS	WBPT (gP/kg/d)	WBPS	Urinary N (mgN/kg/d)	REE (kcals/kg/d)
Subject 1	3.5	3.3	3.3	3.1	35.1	18.1
2	2.9	2.4	3.7	3.3	69.5	29.4
3	5.4	5.1	3.8	3.6	51.6	29.6
4	2.9	2.6	4.7	4.5	51.4	20.4
5	2.3	2.0	4.8	4.5	46.5	29.5
6	4.0	3.4	2.4	2.2	94.1	26.2
Mean	3.5	3.1	3.8	3.5	58.0	25.5
S.D.	1.1	1.1	0.9	0.9	20.9	5.1

Whole Body Protein Turnover: WBPT, Whole Body Protein Synthesis: WBPS, Grams protein per kilogram per day: gP/kg/d, Daily urinary nitrogen excretion derived from 18 h collection taken prior to surgery: Urinary N. Resting energy expenditure: REE, kilocalories per day: kcals/d.

Table 5.2a

Resting energy expenditure and respiratory quotient in healthy subjects

		VCO2 (l/min)	VO2 (l/min)	RQ	REE (kcal/d)
Subject	1	0.152	0.195	0.78	1336
	2	0.246	0.293	0.84	2035
	3	0.206	0.258	0.80	1775
	4	0.168	0.207	0.81	1429
	5	0.199	0.249	0.80	1714
	6	0.275	0.324	0.85	2255
Mean		0.208	0.254	0.81	1757
S.D.		0.046	0.049	0.03	350

VCO2, carbon dioxide production; VO2, oxygen consumption; RQ, respiratory quotient; REE, resting energy expenditure;
REE (kcal/d)= $(3.9VO_2+1.1VCO_2)X1440$
RQ= VCO_2/VO_2

Table 5.3

Liver protein synthetic rates in healthy subjects measured using [15N]glycine

		HFSR(LHP) (%/d)	HFSR(PP) (%/d)	HPS(LHP) (gP/d)	HPS(LHP)/WBPS (%)
Subject	1	10.9	6.8	23.5	9.9
	2	10.5	10.4	21.1	12.7
	3	10.8	7.7	18.9	6.2
	4	11.1	8.3	22.6	12.9
	5	11.1	8.5	18.8	17.1
	6	10.6	6.4	26.6	9.1
Mean		10.8	8.0	21.9	11.3
S.D.		0.3	1.4	2.9	3.8

Hepatic fractional synthetic rate (HFSR) was calculated using either the ^{15}N enrichment of plasma free glycine to represent the precursor pool (PP) or the ^{15}N enrichment of free glycine from the liver homogenate (LHP). The rate of hepatic protein synthesis in grams per day (HPS) was calculated assuming that liver mass is 2% of body weight and that 146g/kg of liver is protein (Geigy Scientific Tables). Hepatic protein synthesis is expressed as a percentage of whole body protein synthesis (HPS(LHP)/WBPS).

Table 5.3a

Wet weights of liver and skeletal muscle biopsies taken from healthy subjects

		Liver (mg)	Muscle (mg)
Subject	1	859	591
	2	425	220
	3	557	541
	4	375	769
	5	482	803
	6	215	726
Mean		486	608
S.D.		216	216

Liver, wedge liver biopsy; Muscle, rectus abdominus biopsy.

Table 5.4

Muscle protein synthetic rates in healthy subjects measured using [15N]glycine

		MFSR(MHP) (%/d)	MFSR(PP) (%/d)	MPS(MHP) (gP/d)	MPS(MHP)/WBPS (%)
Subject	1	1.7	1.1	63.4	25.9
	2	2.9	2.4	100.8	60.8
	3	2.2	1.7	66.5	21.7
	4				
	5	3.7	3.3	108.1	93.1
	6	3.3	3.1	143.0	48.9
Mean		2.8	2.3	96.4	50.1
S.D.		0.8	0.9	32.8	28.9

Muscle fractional synthetic rate (MFSR) was calculated using either the ^{15}N enrichment of plasma free glycine to represent the precursor pool (PP) or the ^{15}N enrichment of free glycine from the muscle homogenate (MHP). The rate of muscle protein synthesis in grams per day (HPS) was calculated assuming that muscle mass is 29.3% of body weight and that 172g/kg of muscle is protein (ICRP, 1975). Muscle protein synthesis is expressed as a percentage of whole body protein synthesis (MPS(MHP)/WBPS).

Table 5.5

Liver protein synthetic rates in healthy subjects measured using [13C]leucine

		HFSR(PP) (%/d)	HPS(PP) (gP/d)	HPS(PP)/WBPS (%)
Subject	1	6.9	14.9	6.5
	2	3.4	6.9	3.0
	3	8.5	14.8	6.9
	4	8.6	17.6	6.0
	5	10.9	18.5	7.0
	6	4.3	10.8	5.7
Mean		7.1	13.9	5.9
S.D.		2.8	4.4	1.5

Hepatic fractional synthetic rate (HFSR) was calculated using either the ^{13}C enrichment of plasma free leucine to represent the precursor pool (PP) as there was insufficient sample to measure the isotopic enrichment of free leucine from the homogenate. The rate of hepatic protein synthesis in grams per day (HPS) was calculated assuming that liver mass is 2% of body weight and that 146g/kg of liver is protein (Geigy Scientific Tables). Hepatic protein synthesis is expressed as a percentage of whole body protein synthesis (HPS(PP)/WBPS).

Table 5.6

Plasma free [15N]glycine and [13C]leucine enrichment of healthy subjects in period prior to biopsy

[^{15}N]glycine		Time to operation -120 min	-60 min	0 min
Subject	1	0.3613	0.4756	0.4614
	2	0.4068	0.4068	0.4311
	3	0.5489	0.4809	0.5428
	4	0.4713	0.4930	0.6239
	5	0.4091	0.3822	0.3841
	6	0.3488	0.4641	0.3895
Mean		0.4243	0.4504	0.4721
S.D.		0.0747	0.0449	0.0942

[^{13}C]leucine		Time to operation -120 min	-60 min	0 min
Subject	1	1.2277	1.5734	1.1389
	2	0.8916	1.1512	1.3739
	3	1.0644	1.4160	1.4419
	4	1.7906	1.0011	0.9922
	5	1.3916	1.3407	1.0134
	6	0.7707	1.4749	1.7186
Mean		1.1894	1.3262	1.2798
S.D.		0.3697	0.2134	0.2833

The measurement of plasma free [^{15}N]glycine and [^{13}C]leucine (atom% excess) in the immediate pre-operative period. The values obtained at each time point for the 6 subjects were not significantly different assessed by analysis of variance (Kruskal-Wallis).

Table 5.7

Plasma free amino acid concentrations in healthy subjects at 5 hours prior to and at the time of operation

	NORMAL [a]		BASELINE		OPERATION		CHANGE
	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	(%)
ASP	-		nd		nd		
GLU	46	13	nd		nd		
ASN	47	9	31.3	7.1	32.7	5.6	4.3
SER	127	29	115.8	19.4	117.8	18.9	1.7
GLN	578	85	261.5	77.4	293.8	86.2	12.4
GLY	300	114	287.5	115.8	288.7	122.4	0.4
THR	154	40	114.7	22.0	115.8	20.1	1.0
HIS	83	14	100.8	10.3	96.0	5.4	-4.8
CIT	35	10	40.7	7.1	38.8	7.8	-4.5
3-MH	-		6.2	1.2	6.2	1.0	0.0
ALA	373	87	329.0	78.6	310.5	72.9	-5.6
TAU	141	57	83.5	23.0	92.2	24.2	10.4
ARG	75	24	94.5	17.9	96.0	16.6	1.6
CAR	-	-	0.0	0.0	0.0	0.0	
ABU	22	8	26.0	4.9	27.5	5.4	5.8
TYR	61	13	52.2	10.0	48.7	7.6	-6.7
VAL	209	31	199.3	27.4	193.7	19.4	-2.8
MET	27	5	22.7	4.4	24.0	5.9	5.9
TRP	50	13	32.0	7.3	29.7	3.7	-7.3
PHE	56	8	52.5	7.3	51.2	5.1	-2.5
ILE	64	13	58.2	6.9	55.0	5.4	-5.4
LEU*	122	23	111.5	15.9	138.3	11.0	24.1
ORN	54	18	51.8	9.6	51.7	7.3	-0.3
LYS	183	34	169.3	26.0	170.0	21.5	0.4
TOTAL AA			2241.0	340.4	2278.2	296.6	1.7

All values in nmol/ml plasma. [a], normal values for plasma amino acid concentrations in female subjects (Geigy Scientific Tables (a). nd, none detected. n=6, * $p<0.05$

Table 5.8

Liver and muscle free amino acid concentrations in healthy subjects

	LIVER		MUSCLE	
	MEAN	S.D.	MEAN	S.D.
ASP	nd		nd	
GLU	53.7	12.9	7.7	2.7
ASN	1.1	0.2	1.1	0.5
SER	8.2	1.2	4.8	2.2
GLN	2.6	1.2	38.8	15.7
GLY	36.0	9.1	6.9	2.7
THR	4.2	1.0	2.4	1.2
HIS	35.6	6.9	1.1	0.7
CIT	0.7	0.5	0.7	0.5
3-MH	0.0	0.0	0.0	0.0
ALA	30.8	7.6	15.6	5.9
TAU	5.7	1.5	15.8	5.6
ARG	1.8	0.7	2.4	1.5
CAR	0.0	0.0	11.0	2.4
ABU	2.4	0.7	0.4	0.2
TYR	1.6	0.5	0.6	0.2
VAL	2.8	0.7	1.4	0.5
MET	11.8	2.4	0.0	0.0
TRP	0.0	0.0	0.0	0.0
PHE	1.7	0.5	0.5	0.2
ILE	1.3	0.2	0.6	0.2
LEU	4.1	1.0	1.5	0.5
ORN	1.9	0.7	0.8	1.0
LYS	2.3	0.5	3.2	1.5
TOTAL AA	210.2	40.4	117.1	44.6

All values in nmol/mg tissue wet weight. n=6. nd, none detected.

Table 5.9

Urinary creatinine and 3-methylhistidine excretion in healthy subjects

		Creatinine* (mmol/vol)	Muscle Mass (kg)	Muscle Protein (gP)	3-MeH (umol/mmol creatinine)
Subject	1	3.4	10.2	1.7	39
	2	5.4	16.3	2.8	20
	3	3.5	10.5	1.8	43
	4	3.6	10.8	1.9	31
	5	2.7	8.1	1.4	33
	6	8.5	25.6	4.4	27
Mean		4.5	13.6	2.3	32
S.D.		2.1	6.5	1.1	8

* 18h urine collection. Whole body muscle mass was calculated on the basis that excretion of 1g urinary creatinine (in 24h) is derived from 20kg muscle (Greystone, 1968) and protein assumed to constitute 172gP/kg muscle mass (ICRP, 1975).

Table 5.10

Liver and Muscle RNA concentration in healthy subjects

		Liver (ug/mg Protein)	Muscle	Liver/Muscle
Subject	1	6.6	0.8	8.2
	2	3.8	0.4	9.5
	3	7.6	-	-
	4	1.6	0.1	16.0
	5	2.2	0.3	7.3
	6	3.4	0.7	4.9
Mean		4.2	0.5	9.2
S.D.		2.4	0.3	4.1

Table 5.11

Comparison of rates of skeletal muscle protein synthesis in normal subjects as determined by different investigators

Author (ref)	Isotope	F.S.R. (%/d) Mean	S.E.M	Comments
Halliday (a)	[^{15}N]lys	3.8	0.4	Sarcoplasmic
		1.4	0.3	Myofibrillar
Rennie (b)	[^{13}C]leu	2.4	0.4	
Halliday (c)	[^{13}C]leu	1.1	0.1	
Garlick (d)	[^{13}C]leu	1.9	0.1	Flooding dose
Shaw (e)	[^{14}C]leu	2.4	0.4	
This work	[^{15}N]gly	2.8	0.3	

(a) Halliday et al., 1975 (b) Rennie et al., 1982 (c) Halliday et al., 1989 (d) Garlick et al., 1989 (e) Shaw et al., 1991.

Table 5.12

Whole body and tissue protein synthetic rate measurements in normal subjects using [15N]glycine

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
Age	70	53	74	67	58	39
Sex	F	F	F	F	F	F
Weight (kg)	74	69	60	70	58	86
[15N]glycine (g/24h)	0.1	0.1	0.1	0.1	0.1	0.1
Nitrogen excreted (g/18h)	1.86	3.53	2.47	2.76	2.16	5.71
Glycine-Tissue (h)	20.3	21.5	20.3	20	20.6	21
End-product (urine ammonia)						
Pre-infusion (AP)	0.36079	0.36203	0.36835	0.37027	0.36365	0.36506
Plateau (AP)	0.40815	0.42327	0.40577	0.42992	0.45637	0.40021
WBPT (gP/kg/day)	3.5	2.9	5.4	2.9	2.3	4
WBPS (gP/kg/day)	3.3	2.4	5.1	2.6	2	3.4
Plasma free glycine						
Pre-infusion (AP)	0.3537	0.37056	0.3774	0.37647	0.37875	0.36914
-2h (APE)	0.36133	0.40685	0.54895	0.47133	0.40906	0.34881
-1h (APE)	0.47559	0.40685	0.48088	0.49301	0.3822	0.46413
0h (APE)	0.46137	0.43115	0.54277	0.62391	0.38409	0.3895
Plateau (APE)	0.46848	0.419	0.51182	0.55846	0.38314	0.42681
Homogenate free glycine						
Skeletal muscle (APE)	0.30799	0.35358	0.39427	*	0.34294	0.40033
Liver (APE)	0.29199	0.41658	0.36761	0.4183	0.29296	0.26008
Hydrolysate glycine						
Skeletal muscle (APE)	0.0045	0.00905	0.00731	*	0.01104	0.01173
Liver (APE)	0.0271	0.0394	0.0337	0.03888	0.0281	0.02423
Fractional Synthetic Rate-homogenate free precursor						
Skeletal muscle (%/day)	1.7	2.9	2.2	*	3.7	3.3
Liver (%/day)	10.9	10.5	10.8	11.1	11.1	10.6
Fractional Synthetic Rate-plasma precursor						
Skeletal muscle (%/day)	1.1	2.4	1.7	*	3.3	3.1
Liver (%/day)	6.8	10.4	7.7	8.3	8.5	6.4

AP atom%, APE atom% excess, Glycine-Tissue (h) duration of glycine infusion prior to biopsy, * sample lost to analysis

Table 5.13

Whole body and tissue protein synthetic rate measurements in normal subjects using [13C]leucine

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
Age	70	53	74	67	58	39
Sex	F	F	F	F	F	F
Weight (kg)	74	69	60	70	58	86
[13C]leucine (mg/h)	65	65	65	65	65	65
Leucine-Tissue (h)	4.8	5.3	5.2	5	5.2	5.8
End-product (breath CO2)						
Breath CO2-pre-infusion (AP)	1.1291	1.1203	1.121	1.1282	1.1212	1.1191
Plateau (APE)	0.0184	0.0144	0.0172	0.0141	0.0243	0.0154
VCO2 (ml/min)	152	475	206	168	199	275
WBPT (gP/kg/day)	3.3	3.7	3.8	4.7	4.8	2.4
WBPS (gP/kg/day)	3.1	3.3	3.6	4.5	4.5	2.2
Plasma free leucine						
Pre-infusion (AP)	1.0935	1.12518	1.11514	1.11537	1.1118	1.11495
-2h (APE)	1.22767	0.89161	1.06438	1.79058	1.39159	0.77074
-1h (APE)	1.57345	1.15119	1.41604	1.00112	1.34068	1.47494
0h (APE)	1.1389	1.3739	1.44187	0.9922	1.01343	1.71863
Plateau (APE)	1.35617	1.26254	1.42895	0.99666	1.17705	1.59678
Hydrolysate leucine						
Liver (APE)	0.01888	0.00932	0.02615	0.0179	0.02763	0.0166
Fractional Synthetic Rate-plasma precursor						
Liver (%/day)	6.9	3.4	8.5	8.6	10.9	4.3

AP atom%, APE atom% excess, Glycine-Tissue (h) duration of glycine infusion prior to biopsy, * sample lost to analysis

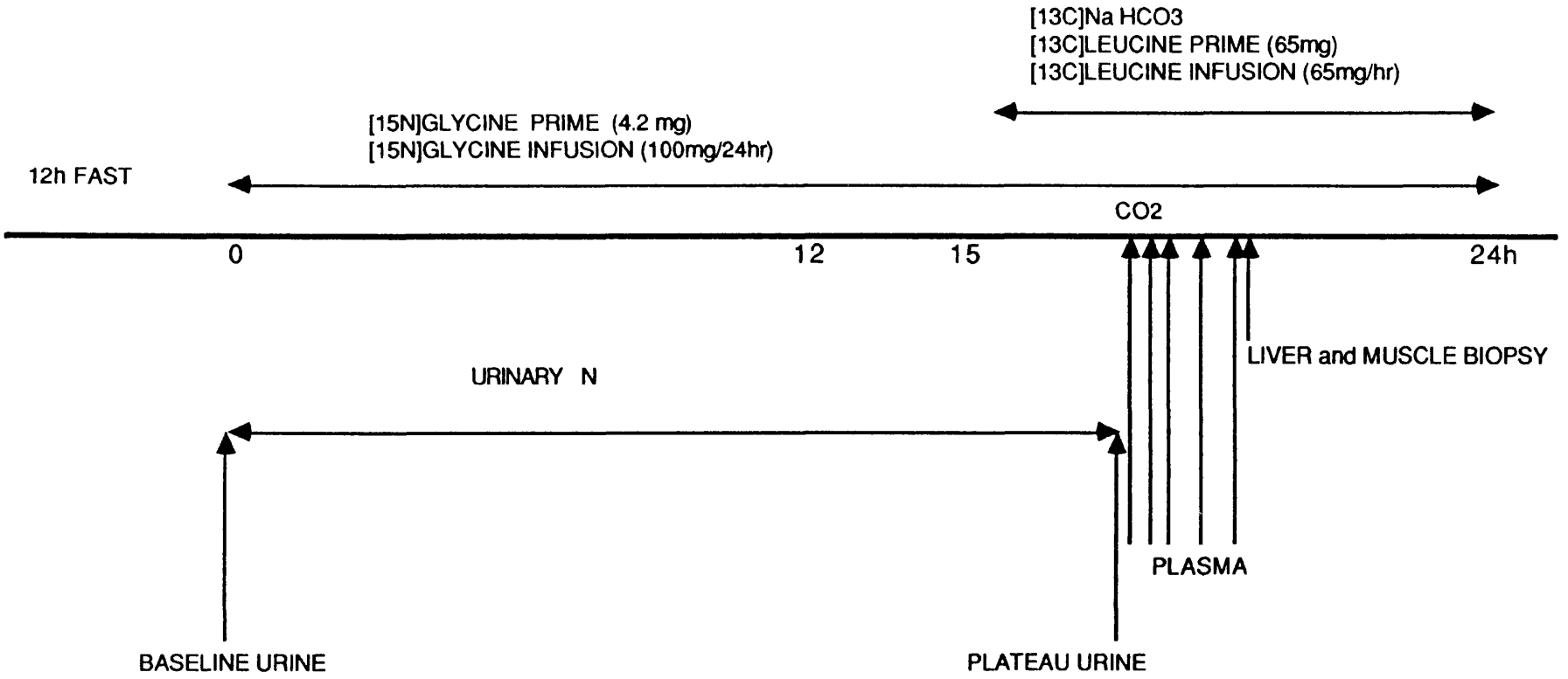


Figure 5.1 Study Protocol

CHAPTER 6: THE SIMULTANEOUS MEASUREMENT OF WHOLE BODY, FIXED LIVER AND MUSCLE PROTEIN SYNTHETIC RATES IN WEIGHT-LOSING CANCER PATIENTS USING [15N]GLYCINE

6.1 Introduction

As discussed in Chapter 1 the majority of cancer patients with progressive disease lose weight, and a proportion become emaciated to the extent that they appear to die primarily from cachexia (Warren, 1932; Inagaki, Rodriguez and Bodey, 1974). The mechanisms which underlie this complex syndrome of nutritional and metabolic upset are poorly understood (Calman, 1982; Fearon and Carter, 1988). One observation which has repeatedly been made is that a proportion of cancer patients with weight loss appear to have inappropriately elevated rates of whole body protein turnover (Jeevanandam et al., 1984; Eden et al., 1984; Inculet et al., 1987; Fearon et al., 1988). Such increased protein turnover might compromise the normal adaptation to semi-starvation and contribute to the accelerated weight loss of the cachectic cancer patient (Eden et al., 1984). However, it is not known which tissues in the body contribute to this elevated protein flux.

The muscle and liver are thought to be the key organs in the regulation of amino acid metabolism and together represent more than 40% of body protein mass in adult man. In addition, studies in animals have suggested that these organs may account for more than 50% of protein turnover in the whole body (Waterlow, Garlick and Millward, 1978). Various studies of body composition and organ volumes in cachectic cancer patients have suggested that muscle mass is markedly reduced whereas the liver mass tends to be preserved (Preston et al., 1987) or even increased (Heymsfield and McManus, 1985). Clearly the

mass of protein of an organ is determined by the balance between synthesis and degradation. If the preserved or even increased hepatic mass of cancer patients were principally the result of enhanced protein synthesis (rather than reduced degradation) then this could mean that the liver was one of the main sites for the apparent increase in whole body protein synthesis.

Rates of protein synthesis measured in the tissues of tumour bearing animals or man have shown a great variety of change. In tumour-bearing rats Norton and coworkers (1981) reported liver protein fractional synthesis rates, measured by [^{15}N]glycine infusion, to be increased whereas, fractional synthetic rates in skeletal muscle were decreased. Pain, Randall and Garlick, (1984) reported similar results in tumour-bearing mice with rates of protein synthesis increased in liver and decreased in skeletal muscle. However in a different mouse model, Emery, Lovell and Rennie, (1984b) reported reduced protein fractional synthetic rates in both liver and muscle. Comparative studies in man are few but, Lundholm and coworkers (1978) have reported that hepatic protein synthesis measured in-vitro with [^{14}C]leucine is increased whereas skeletal muscle protein synthesis in vitro is reduced (Lundholm et al., 1976). A marked reduction in skeletal muscle protein synthesis in cachectic lung cancer patients has also been observed by Emery and coworkers (1984a) in-vivo using [^{13}C]leucine. Such contradictory results may underly methodological problems or may reflect a heterogenous response to malignancy in both animals and man.

With particular reference to hepatic metabolism this may be considered in terms of export and fixed protein components. The majority of cancer patients with advanced disease and weight loss will have an acute phase protein response. These proteins are considered to be of vital

importance to allow recovery following acute injury and to this end it has been suggested that the body will give priority to the synthesis and export of these proteins by the liver. In contrast to hepatic export proteins, little is known about the rate of fixed hepatic protein synthesis. This component of hepatic protein synthesis may be of equal or greater functional importance when compared to the export component yet there has only been one direct measurement of non-export hepatic protein synthesis in patients with cancer (Stein et al., 1978a). Furthermore, there has been no comparison of whole body, liver and muscle protein synthesis in-vivo between normal and weight-losing cancer patients. The aim of the present study was, therefore, to obtain measurements of whole body and tissue protein synthesis rates in weight-losing cancer patients and compare these with the 'normal' values already reported in Chapter 5.

6.2 Materials and methods

6.2.1 Subjects

Six patients with histologically proven colonic adenocarcinoma and hepatic metastasis who had not undergone surgery within the previous 3 months and who had evidence of an acute phase protein response (serum C-reactive protein concentration >10mg/l) were entered into the study. The patients were weighed, wearing light night attire, on beam balance standing scales (Weylux 424, UK.) on the morning of the day that the [^{15}]glycine infusion was commenced. Each patient was questioned carefully about their usual weight and weight loss. This method relied on each patient's subjective impression of their usual body weight and such recall will have a degree of error. However, it has been reported that it is more reliable to estimate weight loss by using the patient's recalled well weight than by using published tables (Morgan, Hill and Burkinshaw, 1980). These patients had been admitted to undergo insertion of an implantable injection system for administration of chemotherapy via the hepatic artery. All individuals were of a performance status 2 or better, defined as 'Ambulatory and capable of all self care but unable to carry out any work; up and about more than 50% of waking hours' (WHO performance status, 1979) 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 severely anaemic. All patients had a normal serum urea and creatinine concentration and although some patients with hepatic metastasis had abnormal liver function tests, none were clinically jaundiced (serum bilirubin <35umol/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 written informed consent.

6.2.2 Experimental design, analytical methods and calculations

These were the same as described in chapters 3,4 and 5 except that only [^{15}N]glycine was used as a tracer to measure whole body, fixed liver and skeletal muscle protein synthesis. The wet weight of the tissues biopsied is given in Table 6.3a.

6.3 Results

The clinical features of the subjects are shown in Table 6.1. In contrast to the normal subjects (reported in Chapter 5) the cancer patients had lost on average 10% of their pre-illness stable weight and this had occurred over a mean period of four months. The mean serum albumin concentration of the cancer patients was 34g/l, below the normal range (36-44g/l) and significantly lower than that of the controls (Chapter 5,Table 5.1, $p<0.05$). The mean body mass index for the cancer patients was 24, within the normal range (20-25) and significantly lower than that of the control group (Table 5.1, $p<0.05$) who were in the overweight range (25-30, Owen, 1988).

Individual rates of whole body protein turnover, whole body protein synthesis, urinary nitrogen excretion and resting energy expenditure are presented in Table 6.2. The mean value for whole body protein turnover and synthesis measured using [^{15}N]glycine was 5.9 and 5.3gP/kg/d respectively which was significantly greater than that of the controls (3.5 and 3.1gp/kg/d, $p<0.01$). Mean rates of whole body protein breakdown exceeded those of whole body protein synthesis by 0.6gP/kg/d in cancer

patients and by 0.4gP/kg/d in the controls. This greater protein loss in the cancer patients was not reflected in statistically higher rates of urinary nitrogen excretion in the cancer patients than in the controls (89.6 $\pm$ 22.0 v 58.0 $\pm$ 8.5mgN/kg/d).

The mean resting energy expenditure in the cancer patients was 27.1kcals/kg/d which was not significantly different from that of the controls (25.5kcals/kg/d :Chapter 5, Table 5.2).

Tissue protein synthesis rates are shown in Table 6.3 and 6.4. The sample from subject 4 was lost during sample preparation (Table 6.4). When the isotopic enrichment of plasma free glycine was taken to represent the enrichment in the precursor pool for protein synthesis the mean values for the liver and muscle protein fractional synthetic rates were 5.6 and 2.9%/d respectively. In contrast, when the isotopic enrichment of free glycine derived from the liver and muscle homogenate was taken to represent the precursor pool enrichment these rates increased to 7.6 and 5.4%/d respectively. It was proposed in Chapter 5 that the homogenate free glycine is more likely to represent the true precursor for protein synthesis. The muscle fractional synthetic rate was 93% higher ($p < 0.05$) and the non-export hepatic fractional synthetic rate was 37% ($p<0.01$) lower in the cancer patients than in the controls. The mean rates of liver and muscle protein synthesis expressed in grams of protein were calculated to be 14.5 and 201gP/kg/d respectively. These rates were calculated using the enrichment of free glycine in the tissue homogenate and assumed that liver weight was 2% of total body weight and that 146g in each kg of liver was protein (Geigy Scientific Tables). Similarly, it was assumed, that muscle weight was 29.3% of total body weight in females and 40% in males and that 172g in each kg of muscle was protein (ICRP 23). When liver and muscle protein synthesis was calculated as a

proportion of whole body protein synthesis the mean values were found to be 4.6 and 62.9% respectively. The isotope enrichment data, from which the whole body and tissue protein fractional synthetic rates were calculated, for [^{15}N]glycine are presented in Table 6.10.

The mean value for liver RNA concentration was 1.2ugRNA/mgProtein and muscle was 2.4ugRNA/mgProtein (Table 6.9). There was no significant difference between the cancer group and the controls (Table 5.10), although there was much greater variation in the results of the cancer group especially in muscle. Therefore, the results are of uncertain value (see section 3.5.3, 5.4).

The intracellular amino acid profile of liver and skeletal muscle are shown in Tables 6.5 and 6.6. The mean total amino acid concentrations were 187.8 and 88.5nmol/mg wet weight respectively. In the comparison with the control group there were significant reductions in asparagine, histidine, citrulline and alanine concentrations ($p<0.05$) in the liver tissue. In the muscle tissue there was a trend towards a reduction in the concentration of most amino acids but this trend did not reach statistical significance. In certain patients it was possible to biopsy the liver tumour. The amino acid profile for the tumour samples (ie. liver tumour) is distinct from that of liver or muscle (Table 6.7). The most notable feature is the proportion of total amino acids made up by the essential amino acids. In normal muscle (8%), cancer muscle (8%), normal liver (13%), cancer liver (19%) and tumour (25%). This is principally due to a decrease in the tissue concentration of the non-essential amino acids in the cancer patients compared with the control group.

Urinary creatinine and 3-Methylhistidine excretion are shown in Table 6.8. The mean creatinine excretion in the 18hr urine collection was 5.4mmol/l and 3-MeH was 27umol/mmol creatinine.

6.4 Discussion

In this study we have examined a homogeneous group of patients with hepatic metastasis from colon cancer. The patients, therefore, had similar tumour type and burden. In order to avoid any confounding metabolic factors associated with severe cachexia or malnutrition, patients were studied when on average they had lost 10% of their pre-illness weight (Table 6.1).

This study demonstrates that patients with hepatic metastasis secondary to colon cancer have rates of whole body protein synthesis which are 70% greater than those observed in non-cancer controls (Chapter 5, Table 5.2). These results are consistent with previous findings (Fearon et al., 1988) and agree with those of other investigators who have observed increased whole body protein synthesis in patients with advanced malignancy (Eden et al., 1984; Jeevanadam et al., 1984). Furthermore, despite a significant increase in whole body protein turnover there was no increase in resting energy expenditure in the cancer group compared to the controls (Chapter 5, Table 5.2). This observation has been made previously in cancer patients with (Fearon et al., 1988) and without weight loss (Melville et al., 1990) and is discussed further in Chapter 7.

In contrast to the whole body measurements, non-export hepatic protein synthesis in cancer patients was reduced by approximately 30% compared to controls, irrespective of whether plasma or liver homogenate free [^{15}N]glycine enrichment was taken to represent the true precursor enrichment (Table 6.3 and Chapter 5, Table 5.3). This reduction in liver protein synthesis in the cancer group is supported by the reduction in mean liver RNA (by about 70%) and is associated with a trend towards reduced tissue free amino acid concentrations (11%) in the cancer group

(Tables 6.9 and 6.5) (Chapter 5, Table 5.10, p=ns). This clearly excludes accelerated synthesis of structural hepatic proteins as a factor which might contribute to the increased protein synthesis in the whole body. Furthermore, these results suggest that any increase in hepatic protein mass associated with malignancy (Heymsfield and McManus, 1985) is likely to be the outcome of decreased protein degradation rather than increased synthesis.

These observations represent only the second in vivo measurements of hepatic non-export protein synthesis in cancer patients and because of the limitations of the first study (Stein et al., 1978) discussed above (see section 5.4) it is not possible to compare the findings. With reference to various animal models our findings are consistent with those of Emery and coworkers (1984b) who demonstrated a reduction in protein synthesis in the liver of thymectomised mice bearing the XK1 tumour. This is an important model of human cancer cachexia since the XK1 tumour is human in origin and induces weight loss when tumour mass is similar to that at which weight loss occurs in man (ie. <5% total body weight Stein, 1982a). In contrast, Norton et al, (1981) reported increased liver protein synthesis in rats bearing a methylcholanthrene induced sarcoma. However, the fractional synthetic rate was calculated from ^{15}N enrichment of the amino nitrogen in the tissue free homogenate and Kjeldahl digest of the protein bound fraction. Also, there was no weight-loss in the cancer group that they studied. Pain and coworkers (1984) reported increased total hepatic protein synthesis in mice bearing a rodent ascites tumour. However, in this model the tumour mass accounted for 60% of total body weight and the situation cannot be compared with that in man. Lundholm and coworkers (1978) reported an increased incorporation rate of amino acids into hepatic proteins in sarcoma-bearing mice as well as in liver

tissue from cancer patients. However, the latter study involved using isolated tissue samples incubated in vitro and the results obtained may not be physiologically meaningful. Moreover, the mouse in vivo studies performed by Lundholm and coworkers were limited to measurements of incorporation of radioactively labelled amino acids into protein, without consideration of the precursor pool specific activity, so that the absolute rates of protein synthesis could not be calculated.

In the present study, it is clear from the liver and muscle biopsies that there was a decrease in the concentration of the non-essential amino acids (principally alanine) in the cancer group. The reduction in alanine concentration was statistically significant for liver ($p<0.05$) but not for muscle ($p=0.17$). It is possible that the decrease in alanine concentration in these tissues reflects an increased demand for alanine as a glucose precursor (Felig et al., 1969). There have been repeated clinical and experimental observations of abnormal carbohydrate metabolism in cancer patients (Kern and Norton, 1988). More specifically, a decrease in peripheral glucose disposal (Lundholm et al., 1978) and an increase in basal hepatic glucose production (Shaw and Wolfe, 1986) have been reported in cancer patients. These reports are consistent with increased alanine utilisation in liver and muscle and may explain the reduction in tissue alanine concentration in the cancer group.

In the present study there was a mean increase of 26% or 93% in skeletal muscle protein synthesis rates when compared with the controls depending on whether plasma or muscle homogenate free [^{15}N]glycine enrichment was taken to represent the true precursor enrichment (Table 6.4 and Chapter 5, Table 5.4). In contrast, several investigators have reported reduced rates of protein synthesis in the skeletal muscle of humans (Lundholm et al., 1976; Emery et al., 1984a) and animals (Norton

et al., 1981; Emery, Lovell and Rennie, 1984b) with cancer cachexia. However, all of these studies used leucine as a tracer with the exception of Norton et al, (1981) who used glycine but measured the incorporation of ^{15}N into the amino nitrogen of muscle protein, and therefore the results of the latter study are not directly comparable with that of the present. Furthermore, the subjects studied by other investigators were severely cachectic and presumably had severe muscle wasting. In contrast, the cancer patients examined in the present study had sustained only 10% weight loss and appeared to have a normal muscle mass when calculated from urinary creatinine excretion (with the exception of subject 2, Table 6.8). This is in accord with the normal body mass index measured (Table 6.1) and body composition studies which suggest that 10% documented weight loss in cancer patients is associated with a small reduction in lean body mass (more than half of lean body mass is skeletal muscle) of approximately 5% (Cohn et al., 1981; Shizgal, 1985).

When the mean increase in skeletal muscle protein synthesis rate (93%) is taken with a mean reduction in non-export hepatic protein synthesis rate of 37% and compared with an mean increase of 71% in whole body protein synthesis rate, it appears that the increase in skeletal muscle protein synthesis is sufficient to account for the increase in whole body protein synthesis (see Table 7.3). An increase in skeletal muscle protein synthesis would be unlikely in the presence of the marked reduction in intracellular amino acid concentration which was observed (Table 6.6). In addition, there was no increase in skeletal muscle breakdown as measured by 3-Methylhistidine excretion (Table 6.8 and Chapter 5, Table 5.9) (Young and Munro, 1978; Ballard and Thomas, 1983; Rennie and Millward, 1983; Long et al., 1988). An increase in muscle protein synthesis without an accompanying increase in muscle

protein breakdown would result in increased muscle mass which is clearly not the case in patients with weight-loss.

Such findings suggest that perhaps the incorporation of [^{15}N]glycine may not give a good estimate muscle protein synthesis in cancer patients. Furthermore, the exact relationship between kinetic parameters measured by tracer amino acid infusion and body protein kinetics remains unclear. The fact that that the 70% increase in whole body protein synthesis was not accompanied by an increase in resting energy expenditure suggests there may be a breakdown in the relationship of glycine flux to whole body and skeletal muscle protein synthesis in cancer patients. Possible mechanisms whereby this could occur are discussed in Chapter 7.

Table 6.1

Characteristics of cancer patients

		Age (yrs)	Sex	Weight (kg)	Height (cm)	BMI	Weight loss(%)	Albumin (g/l)
Subject	1	54	M	63	168	22	19	34
	2	78	F	52	157	21	15	24
	3	59	M	67	175	22	7	39
	4	42	M	77	170	27	8	35
	5	68	M	72	168	26	6	39
	6	62	M	65	165	24	7	31
Mean		61		66	167	24	10	34
S.D.		12		9	6	2	5	6

BMI, Body Mass Index=weight(kg)/[height(m)]^2

Table 6.2

Whole body protein kinetics and resting energy expenditure in weight-losing cancer patients.

		[^{15}N]glycine WBPT (gP/kg/d)	WBPS (gP/kg/d)	Urinary N (mgN/kg/d)	REE (kcals/kg/d)
Subject	1	5.4	5.0	58.7	29.4
	2	7.4	7.3	26.9	24.8
	3	3.5	2.8	125.3	24.3
	4	5.6	5.0	97.4	31.3
	5	5.6	5.3	55.5	25.5
	6	7.6	6.5	173.8	27.0
Mean		5.9	5.3	89.6	27.1
S.D.		1.5	1.5	53.8	2.8

Whole Body Protein Turnover: WBPT, Whole Body Protein Synthesis: WBPS, Grams protein per kilogram per day: gP/kg/d, Daily urinary nitrogen excretion derived from 18 h collection taken prior to surgery: Urinary N.

Table 6.2a

Resting energy expenditure and respiratory quotient of weight-losing cancer patients

		VCO2 (l/min)	VO2 (l/min)	RQ	REE (kcal/d)
Subject	1	0.221	0.267	0.83	1850
	2	0.156	0.186	0.84	1291
	3	0.192	0.236	0.81	1630
	4	0.281	0.350	0.80	2411
	5	0.233	0.262	0.89	1840
	6	0.203	0.256	0.79	1759
Mean		0.214	0.260	0.83	1797
S.D.		0.042	0.053	0.04	365

VCO2, carbon dioxide production; VO2, oxygen consumption; RQ, respiratory quotient; REE, resting energy expenditure;
REE (kcal/d)= $(3.9VO_2+1.1VCO_2)X1440$
RQ= VCO_2/VO_2

Table 6.3

Liver protein synthetic rates in weight-losing cancer patients measured using [15N]glycine

		HFSR(LHP) (%/d)	HFSR(PP) (%/d)	HPS(LHP) (gP/d)	HPS(LHP)/WBPS (%)
Subject	1	7.8	6.2	14.3	4.5
	2	9.5	6.7	14.4	3.8
	3	8.0	4.6	15.6	8.3
	4	9.8	8.8	22.0	5.7
	5	6.2	3.4	13.0	3.4
	6	4.0	3.6	7.6	1.8
Mean		7.6	5.6	14.5	4.6
S.D.		2.2	2.1	4.6	2.2

Hepatic fractional synthetic rate (HFSR) was calculated using either the ^{15}N enrichment of plasma free glycine to represent the precursor pool (PP) or the ^{15}N enrichment of free glycine from the liver homogenate (LHP). The rate of hepatic protein synthesis in grams per day (HPS) was calculated assuming that liver mass is 2% of body weight and that 146g/kg of liver is protein (Geigy Scientific Tables). Hepatic protein synthesis is expressed as a percentage of whole body protein synthesis (HPS(LHP)/WBPS).

Table 6.3a

Wet weights of liver and skeletal muscle biopsies taken from weight-losing cancer patients

		Liver (mg)	Muscle (mg)
Subject	1	226	708
	2	246	414
	3	590	791
	4	412	342
	5	415	975
	6	615	411
Mean		417	607
S.D.		164	255

Liver, wedge liver biopsy; Muscle, rectus abdominus biopsy.

Table 6.4

Muscle protein synthetic rates in weight-losing cancer patients measured using [15N]glycine

		MFSR(MHP) (%/d)	MFSR(PP) (%/d)	MPS(MHP) (gP/d)	MPS(MHP)/WBPS (%)
Subject	1	3.9	2.3	169	54
	2	5.9	2.0	155	41
	3	6.5	3.0		
	4				
	5	6.1	3.4	322	84
	6	4.7	3.2	210	50
Mean		5.4	2.8	214	57
S.D.		1.1	0.6	76	19

Muscle fractional synthetic rate (MFSR) was calculated using either the ^{15}N enrichment of plasma free glycine to represent the precursor pool (PP) or the ^{15}N enrichment of free glycine from the muscle homogenate (MHP). The rate of muscle protein synthesis in grams per day (MPS) was calculated assuming that muscle mass is 29.3% (females) and 40% (males) of body weight and that 172g/kg of muscle is protein (IRCP, 1975). Muscle protein synthesis is expressed as a percentage of whole body protein synthesis (MPS(MHP)/WBPS).

Table 6.5

Liver free amino acid concentrations in normal subjects compared with those of weight-losing cancer patients

	CONTROL LIVER (nmol/mg) MEAN	S.D.	CANCER LIVER (nmol/mg) MEAN	S.D.	% CHANGE
ASP	nd		nd		nd
GLU	53.7	12.9	51.6	12.0	-3.8
ASN	1.1	0.2	2.1	0.7	87.2*
SER	8.2	1.2	8.8	1.9	7.0
GLN	2.6	1.2	5.6	4.7	113.4
GLY	36.0	9.1	30.4	9.1	-15.6
THR	4.2	1.0	4.1	1.2	-0.9
HIS	35.6	6.9	19.7	12.2	-44.6*
CIT	0.7	0.5	0.1	0.2	-91.3*
3-MH	0.0	0.0	0.0	0.0	
ALA	30.8	7.6	19.5	7.8	-36.6*
TAU	5.7	1.5	5.8	2.4	2.3
ARG	1.8	0.7	1.4	0.7	-22.3
CAR	0.0	0.0	0.0	0.0	
ABU	2.4	0.7	2.7	1.0	10.7
TYR	1.6	0.5	1.8	0.5	11.1
VAL	2.8	0.7	3.0	1.0	4.8
MET	11.8	2.4	17.0	6.6	44.8
TRP	0.0	0.0	0.2	0.5	
PHE	1.7	0.5	1.8	0.7	4.5
ILE	1.3	0.2	1.4	0.5	6.1
LEU	4.1	1.0	3.5	1.0	-13.1
ORN	1.9	0.7	3.0	1.5	58.1
LYS	2.3	0.5	4.3	1.7	86.2
TOTAL AA	210.2	40.4	187.8	46.3	-10.7

n=6, * $p<0.05$. nd, none detected.

Table 6.6

Muscle free amino acid concentrations in normal subjects compared with those of weight-losing cancer patients

	CONTROL MUSCLE (nmol/mg)		CANCER MUSCLE (nmol/mg)		
	MEAN	S.D.	MEAN	S.D.	% CHANGE
ASP	nd		nd		
GLU	7.7	2.7	8.7	2.9	12.9
ASN	1.1	0.5	1.1	0.5	-0.1
SER	4.8	2.2	3.2	0.2	-33.6
GLN	38.8	15.7	31.2	5.6	-19.6
GLY	6.9	2.7	4.7	1.0	-32.1
THR	2.4	1.2	1.8	0.5	-26.9
HIS	1.1	0.7	0.2	0.5	-82.5
CIT	0.7	0.5	0.3	0.2	-57.8
3-MH	0.0	0.0	0.0	0.0	
ALA	15.6	5.9	10.9	2.5	-30.5
TAU	15.8	5.6	12.4	3.7	-21.5
ARG	2.4	1.5	1.7	0.7	-30.4
CAR	11.0	2.4	6.8	3.9	-38.0
ABU	0.4	0.2	0.3	0.2	-26.3
TYR	0.6	0.2	0.3	0.2	-41.1
VAL	1.4	0.5	1.0	0.2	-29.4
MET	0.0	0.0	0.3	0.2	
TRP	0.0	0.0	0.1	0.2	
PHE	0.5	0.2	0.3	0.2	-23.7
ILE	0.6	0.2	0.3	0.2	-46.4
LEU	1.5	0.5	0.7	0.5	-49.0
ORN	0.8	1.0	0.0	0.0	-100.0
LYS	3.2	1.5	2.3	1.0	-28.4
TOTAL AA	117.1	44.6	88.5	16.4	-24.4

n=6. nd, none detected.

Table 6.7

Tumour free amino acid concentrations in weight-losing cancer patients

	CANCER PATIENTS TUMOUR (nmol/mg)	
	MEAN	S.D.
ASP	nd	
GLU	30.9	26.6
ASN	1.1	0.6
SER	5.3	2.4
GLN	0.1	0.2
GLY	15.2	8.4
THR	2.9	1.2
HIS	2.4	2.8
CIT	0.4	0.4
3-MH	0.0	0.0
ALA	15.4	11.8
TAU	15.7	6.0
ARG	1.0	0.6
CAR	0.0	0.0
ABU	0.9	0.8
TYR	1.6	1.0
VAL	3.0	2.0
MET	14.0	10.2
TRP	0.0	0.0
PHE	1.6	1.2
ILE	1.6	1.2
LEU	3.5	2.4
ORN	1.2	1.4
LYS	3.4	1.6
TOTAL AA	121.2	79.0

n=4. nd, none detected.

Table 6.8

Urinary creatinine and 3-methylhistidine excretion in weight-losing cancer patients.

		Creatinine* (mmol/vol)	Muscle Mass (kg)	Muscle Protein (gP)	3-methylhistidine (umol/mmol creatinine)
Subject	1	3.4	10.2	1.7	33
	2	0.9	2.7	0.5	20
	3	6.9	20.8	3.6	55
	4	7.7	23.2	4.0	16
	5	5.2	15.7	2.7	19
	6	10.7	32.2	5.5	21
Mean		5.8	17.5	3.0	27
S.D.		3.4	10.3	1.8	15

* 18h urine collection. Whole body muscle mass was calculated on the basis that excretion of 1g urinary creatinine (in 24h) is derived from 20kg muscle (Greystone, 1968) and protein assumed to constitute 172gP/kg muscle mass (ICRP, 1975).

Table 6.9

Liver and muscle RNA concentrations in weight-losing cancer patients.

		Liver (ug/mg Protein)	Muscle	Liver/Muscle
Subject	1	0.4	0.08	5.0
	2	11.7		
	3	0.2	2.5	0.08
	4	3.5		
	5	0.3	6.7	0.04
	6	1.2	0.2	6.0
Mean		2.9	2.4	2.8
S.D.		4.5	3.1	3.2

Table 6.10

Whole body and tissue protein synthetic rate measurements in weight-losing cancer patients using [15N]glycine

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6
Age	54	78	59	42	68	62
Sex	M	F	M	M	M	M
Weight (kg)	63	52	67	77	72	65
[15N]glycine (g/24h)	0.1	0.1	0.1	0.1	0.1	0.1
Nitrogen excreted (g/18h)	2.78	1.05	6.27	5.59	3.02	8.5
Glycine-Tissue (h)	21	23.3	19.6	21.3	20.7	23.5
End-product (urine ammonia)						
Pre-infusion (AP)	0.36449	0.36551	0.36934	0.36581	0.36464	0.36551
Plateau (AP)	0.40014	0.39696	0.42075	0.39423	0.39473	0.39002
WBPT (gP/kg/day)	5.4	7.4	3.5	5.6	5.6	7.6
WBPS (gP/kg/day)	5	7.3	2.8	5	5.3	6.5
Plasma free glycine						
Pre-infusion (AP)	0.36912	0.36817	0.37002	0.36711	0.36942	0.36835
Plateau (APE)	0.25939	0.80213	0.50285	0.19446	0.39482	0.39504
Homogenate free glycine						
Skeletal muscle (APE)	0.15458	0.26862	0.24763	*	0.2066	0.26999
Liver (APE)	0.20732	0.57148	0.29013	0.17501	0.21715	0.35159
Hydrolysate glycine						
Skeletal muscle (APE)	0.00511	0.01556	0.01291	*	0.01157	0.01228
Liver (APE)	0.01407	0.0528	0.01902	0.01528	0.01154	0.01371
Fractional Synthetic Rate-homogenate free precursor						
Skeletal muscle (%/day)	3.9	5.9	6.1	*	6.5	4.7
Liver (%/day)	7.8	9.5	8	9.8	6.2	4
Fractional Synthetic Rate-plasma precursor						
Skeletal muscle (%/day)	2.3	2	3	*	3.4	3.2
Liver (%/day)	6.2	6.7	4.6	8.8	3.4	3.6

AP atom%, APE atom% excess, Glycine-Tissue (h) duration of glycine infusion prior to biopsy, * sample lost to analysis

CHAPTER 7: GENERAL DISCUSSION OF CLINICAL STUDIES

7.1 Introduction

It has been reported that in cancer patients with and without weight-loss there is an increase in whole body amino acid flux (Table 7.2). Within the assumptions of the two pool model for protein metabolism (Picou and Taylor-Roberts, 1969), this would suggest a significant increase in whole body protein turnover. Nevertheless, the organs or tissues that bring about this increase in protein turnover are unknown. Several investigators have proposed that the liver might be one of the principal organs to contribute to increased protein turnover in the cancer host (Kern and Norton, 1988). However, in an attempt to discover the site of such increased protein synthesis it has been demonstrated in this thesis that at least in the liver of patients with advanced colon cancer and weight-loss, non-export protein synthesis is reduced by 30%. It has been proposed that more than 50% of protein synthesis in the liver is contributed by non-export protein components (Waterlow, Garlick and Millward, 1978g). Thus, if the liver were to account for the major part of the apparent increase in protein synthesis in cancer patients then this would imply that hepatic export protein synthesis was increased some four fold. However, in a chronic wasting state it would seem unlikely that liver export protein synthesis could increase to such an extent.

The fractional synthetic rate of skeletal muscle protein has been reported to be 18-25% of the rate at which this process occurs in the liver, depending on which mammal is studied (rat 25%, pig 18%; Waterlow, Garlick and Millward, 1978b). Moreover, several studies have suggested that protein synthesis in this tissue is markedly decreased in the cachectic cancer host (Emery et al., 1984a; Lundholm et al., 1976).

However, from studies presented in this thesis it would appear that protein synthesis in the skeletal muscle of the cancer host may be markedly increased (Chapter 6) and might, therefore account for the greater part of the increase in whole body protein metabolism. In view of these contradictory results it is the aim of this Chapter to examine critically the nature of both the accelerated whole body tracer flux and increased glycine incorporation into the skeletal muscle of the cancer patients included in the present study.

7.2 Whole body protein synthesis

7.2.1 Comparison with other published work

From the studies detailed in Chapters 5 and 6 there was on average a 69% increase in whole body protein turnover and a 71% increase in whole body protein synthesis in the cancer patients compared to the controls. These findings are in agreement with a number of studies in the literature where whole body protein turnover has been measured using [^{15}N]glycine in cancer patients with reference to a control group in the post absorptive state (Table 7.2). It is clear from other studies (Table 7.2) that the measurements in normal subjects can vary considerably, ranging from 1.9 to 3.9gP/kg/d depending on the controls, type of tracer used and, with [^{15}N]glycine, the end-product used (see section 2.3). Furthermore, in cancer patients the variation is large despite attempts to study homogeneous groups. However, there is good agreement in the results of different studies when the same isotopically labelled tracer has been used. Inculet et al. (1987) and Melville et al. (1990) using [^{13}C]leucine reported an increase in whole body protein turnover of 15% and 19% respectively when weight stable cancer patients were compared with weight-stable

controls. With [^{15}N]glycine more substantial increases have been reported when weight-losing cancer patients have been compared with weight-stable controls (Norton, Stein and Brennan, 1981; Kien and Camitta, 1983; Ward et al., 1985; Fearon et al., 1988; see Table 7.2). In contrast, Jeevanandam and coworkers (1984) and Fearon and colleagues (1988) using [^{15}N]glycine reported smaller increases (see Table 7.2) in whole body protein turnover when weight-losing controls were compared with weight-losing cancer patients.

Whatever the final magnitude of the change, if whole body protein synthesis rather than simple tracer flux were increased substantially then the energy cost of this might be expected to give rise to an increase in host resting energy expenditure. However, in the present study (Chapters 5 and 6) and those in the literature, in which simultaneous measurements of energy expenditure have been made, no relationship has been demonstrated between resting energy expenditure and the increase in whole body protein turnover (Fearon et al., 1988; Melville et al., 1990).

It is of further interest that when whole body protein synthesis has been measured in cancer patients in the fed state rather than in the fasted state, several investigators have failed to demonstrate a significant difference when compared with controls (Emery et al., 1984a; Glass, Fern and Garlick, 1983). The contradictory results obtained by Emery and coworkers (1984a) might be explained by the heterogeneous tumour type of the group studied since they had extensive disease and documented weight loss (as had the group in the present study). However, the contradictory results obtained by Glass and coworkers (1983) were unlikely to be due to different tumour type since a homogenous group were studied. These patients differed in that the stage of disease (Dukes A, C) was not as advanced as those in the present study (Dukes D), and

approximately half of the group studied by Glass and coworkers had suffered weight loss. Alternatively, the data from these two studies (Emery et al., 1984a; Glass, Fern and Garlick, 1983) may simply indicate that the rate of protein turnover responds to feeding by a smaller amount in cancer patients than normal individuals. However, in the light of work carried out in cancer patients showing a normal response to feeding (Kern and Norton, 1988) this appears unlikely. Furthermore, Melville et al. (1990) reported that weight-stable cancer patients had a similar protein turnover response to feeding as controls. Therefore, it would appear that the extent of the increase in whole body protein turnover associated with cancer may be reduced in the fed state. The differences between cancer patients in the fed and fasted state may also reflect the limitations of the amino acid kinetic model which has been used to obtain values for whole body protein turnover and synthesis.

7.2.2 Limitations of the model

[^{15}N]glycine has been used extensively as a tracer for measuring whole body protein synthesis rates in health and disease. There have been a number of studies by different groups which have supported the use of [^{15}N]glycine for turnover studies (Picou and Taylor-Roberts, 1969; Golden and Waterlow, 1977; Stein et al., 1980). Stein and coworkers (1980) validated the use of [^{15}N]glycine for whole body protein synthesis measurements by obtaining similar results using two different methods. They compared, in rats, the indirect method of Picou and Taylor-Roberts (1969), which involves measuring urinary [^{15}N]urea enrichment at plateau, with the direct method of Garlick, Millward and James (1973) using the homogenised whole rat to determine ^{15}N tissue incorporation. Although this approach can be criticised for not giving accurate ('absolute correct')

tissue protein fractional synthetic rates (see section 2.5) nevertheless the assumptions about the distribution of the ^{15}N label in both methods are essentially the same and therefore allow a meaningful comparison.

The concept of a single value to represent the sum of all rates of protein synthesis in the individual tissues remains an abstract concept dependent on the assumptions of the kinetic model used to derive such a value. Although [^{15}N]glycine has been the subject of more investigations than any other isotopically labelled amino acid, all the validation experiments have been done on healthy, non-stressed animals or subjects (Golden and Waterlow, 1977; Waterlow and Jackson, 1981; Waterlow, Golden and Garlick, 1978; Stein et al., 1976b; Stein et al., 1980; Stein et al., 1982b; Taruvinga, Jackson and Golden, 1979). Although it is likely that measured rates in the normal range (2-4gP/kg/day) are reasonable estimates of whole body protein turnover, the same does not necessarily hold for protein turnover rates derived from single amino acid kinetic studies in patients with disease states.

Studies of metabolically stressed subjects have documented elevated (up to two fold) turnover rates in trauma, burns, sepsis and cancer cachexia (Birkahn et al., 1981; Kien et al., 1978; Long et al., 1977; Jeevanandam et al., 1984). However, it must be stressed that such changes are derived from the flux of a single amino acid and a simple model of protein metabolism. It is only when a model of human protein metabolism is applied, such as that described by Picou-Taylor Roberts (1969), that a synthesis rate can be estimated. This model describes protein metabolism in terms of two interrelated pools; a protein pool and an amino acid pool. This highly simplified model seems to apply well in normal and moderately stressed states, but if nitrogen kinetics in the body are greatly disturbed, as is likely in patients that are metabolically stressed

(i.e displaying an acute phase protein response), then most of the assumptions of the model are called into question. One of the fundamental assumptions of the model is that there is a single amino acid pool within the body. Therefore, the existence of more than one amino acid pool, resulting in the labelled amino acid being at different enrichments in each pool (precursor compartmentation) is incompatible with the model.

There is some evidence, (following examination of ^{15}N enrichment in nitrogenous end-products), which supports compartmentation of amino acids at the site of end-product formation in normal (Fern, Garlick and Waterlow, 1985) and metabolically stressed patients (Stein et al., 1983; Taggart et al., 1991). James et al., (1976) reported on theoretical grounds that [^{15}N]glycine may overestimate the contribution of the liver. Animal experiments have also shown compartmentation of glycine-nitrogen metabolism when the amino acid intake is restricted (Stein et al., 1976a). Further evidence comes from the work of Taruvinga, Jackson and Golden, (1979) who reported that, when [^{15}N]-labelled branched chain amino acids were used for whole body protein synthesis measurements, spuriously high rates were found.

In the studies carried out in Chapters 5 and 6 the increase in whole body protein turnover was due to the reduced urinary ammonia enrichment in the cancer group indicating that there was less transfer of [^{15}N] from the administered glycine to the urinary ammonia precursor pool. It may be that if there is compartmentation of the amino acid pools in this group that the urinary ammonia becomes disproportionately derived from a relatively unenriched pool (possibly peripheral tissue). Previous studies have, however, confirmed an elevated rate of tracer flux whether urea or ammonia is used as the end-product of nitrogen metabolism (Fearon et al., 1988). Although there appears to be a greater increase in the rate of tracer

flux when ammonia compared with urea is used as an end-product of nitrogen metabolism (see section 2.3, Table 7.2).

Another assumption of this simplified model of protein metabolism is that the particular labelled amino acid is involved in the synthesis of a mixture of proteins which does not change in the disease state. However, there is evidence that this is not the case with glycine in the metabolically stressed individual (Grimble, 1990). For example, the metabolically interrelated amino acids (glycine, serine and methionine) occur in high concentrations in many of the proteins synthesised in increased amounts as part of the inflammatory response (Grimble, 1990; Table 7.8). The incorporation of label associated with these amino acids may elevate the whole body protein synthesis estimate using [^{15}N]glycine (Matthews et al., 1981b). An increase in the fractional synthetic rate of fibrinogen (one of the proteins synthesised in increased amounts during inflammation) in patients with gastrointestinal cancer (26%/d) compared with normal subjects (15%/d) has been reported (Stein et al., 1978b; Stein et al., 1978a). Using [^{15}N]glycine to measure simultaneously whole body protein and fibrinogen synthesis rates Stein and coworkers (1990) have reported that the reduction in whole body protein synthesis rates of AIDS patients was accompanied by a reduction in fibrinogen synthesis compared with controls. Other proteins may also be of importance such as collagen. Indeed, when [^{15}N]glycine was used as a tracer for the determination of whole body protein turnover in small preterm babies, very high protein synthesis rates were measured (Plath et al., 1985). This was attributed to a high synthesis rate of structural protein which (because of the composition of collagen: 33% of amino acid residues being glycine) was removing a large amount of glycine from the blood and bringing about the apparent high protein synthesis rates.

The metabolism of amino acids is generally interpretated in the light of the metabolism of protein. However, glycine acts as a precursor for the synthesis of a variety of physiological important compounds which include nucleic acids, porphyrins, hippurate and creatine. The most important, quantitatively, is creatine which can account, depending on diet and nutritional status, for approximately 5% of glycine flux (Reeds, 1981; Neuberger, 1981). Since the sole breakdown product of creatine is creatinine and the conversion of creatine and creatine phosphate to creatinine is non-enzymic and therefore relatively constant, then the urinary creatinine excretion gives an indirect measure of body creatine content. In these studies, urinary creatinine excretion was similar in both the control and cancer groups (Tables 5.9, 6.8) and therefore there is little evidence of markedly altered creatine synthesis.

In summary, from the work presented in this thesis and that in the literature it is clear that the presence of a tumour brings about an increase in whole body glycine flux. However, it is also clear that several assumptions of the model may not allow one to equate such increased glycine flux to an equivalent increase in whole body protein turnover in the weight-losing cancer patient.

7.3 Liver protein synthesis

There has been some controversy whether total liver protein synthesis, measured by labelled amino acids, is increased (Lundholm et al., 1978) or decreased (Emery, Lovell and Rennie, 1984) in cancer cachexia. Recently, Shaw and coworkers (1991), using [^{14}C]leucine and assuming that the specific activity of plasma free leucine represents the true precursor activity for protein synthesis, reported that the fractional synthetic rates for total liver protein synthesis was approximately 29%/d in

a group of weight-losing cancer patients (mean weight loss, 18%). Furthermore, they derived a value for total liver protein fractional synthetic rate (from the albumin synthetic rate) of approximately 21%/d for patients with no cancer and concluded that liver protein synthesis was significantly higher in the weight-losing cancer patients than in the non-cancer weight stable patients (Shaw et al., 1991). However, the multiple assumptions involved in reaching such a conclusion bring its validity into doubt.

The nutritional status of the patient or animal is a complicating factor in such measurements. In 1959 Munro and Clark measured the RNA content in hepatoma and host liver in rats fed a 25% protein diet compared with animals fed an isocaloric, protein free diet (Munro and Clark, 1959). They reported a decrease in RNA content of host liver in rats ingesting the protein free diet suggesting that total liver protein synthesis was decreased. Ota et al., (1977) measured liver protein content and found similar results in Buffalo rats with Morris hepatomas. In contrast, another study of tumour-bearing rats given different intravenous feeds showed that those on low protein or low calorie diets had increased liver protein synthesis but, those on a standard protein and calorie diet had no change in liver protein synthesis (Oram-Smith et al., 1977). Therefore, within the limits of extrapolating tissue RNA and protein contents to actual rates of protein synthesis it is clear that the nutritional status of the animal, and presumably man, may be an important factor in determining whether liver protein synthesis is increased or decreased in cancer. The patients studied in the present thesis had no protein and minimal calorie intake for nearly 36h prior to the measurement being made. However, it is not clear from the above studies whether this would have significantly altered the synthetic rate of non-export liver protein.

The effect of increasing tumour burden on host liver energy status has been studied using P-31 nuclear magnetic resonance spectroscopy in rats inoculated with a non-metastastic methylcholanthrene-induced sarcoma (Schneeberger et al., 1989). This study demonstrated that increasing tumour burden results in early, ongoing, depletion of energy stores as reflected by increasing [Pi]/[ATP] ratio in the liver. It is unlikely that increased liver protein synthesis could be maintained against such a background of reduced energy stores.

In summary, it would appear that the finding of reduced non-export liver protein synthesis in patients with cancer cachexia (characterised by decreased nutrient intake) is in accord with the majority of published work. The question of whether hepatic export protein synthesis rates are changed remains unresolved.

7.4 Muscle protein synthesis

It has been reported that the fractional synthetic rate in skeletal muscle protein is reduced in weight-losing cancer patients compared with normal subjects (Lundholm et al., 1976; Emery et al., 1984a). This finding has been supported by work in some animal models of cancer cachexia (Norton et al., 1981; Emery, lovell and Rennie, 1984b) but not in others (Plumb et al., 1991). It has recently been reported by Shaw and coworkers (1991) that muscle protein fractional synthetic rates are increased in weight-losing cancer patients compared with weight-stable non-cancer patients. Therefore, there is some support for the apparent increased muscle protein synthetic rates reported in the present study.

In order to compare the present study with other reports of muscle protein synthesis rates in normal subjects and weight-losing cancer patients it is necessary to consider details of the various methodologies

that may effect the measured rate. The values for muscle protein fractional synthetic rates based on different tracer amino acids and precursors, in a number of studies are presented (if shown in the manuscript) in Table 7.9. With reference to the muscle protein fractional synthetic rate in normal subjects (see Table 7.9), it is clear that there is considerable variation in the rates reported. This variation may firstly be due to which substrate enrichment is taken to best represent that of aminoacyl-tRNA (for discussion see section 2.4), e.g., plasma alpha-ketoisocaproate (indicative of intracellular leucine enrichment; Halliday et al., 1988) or plasma leucine (Shaw et al., 1991). Further variation may depend upon whether a flooding dose (Garlick et al., 1989) or continuous infusion (Halliday et al., 1988) protocol is used to measure the protein fractional synthetic rate (Table 7.9). However, such differences in measured muscle protein fractional synthetic rate with the two methods may be due to choice of alpha-ketoisocaproate rather than muscle free leucine as the precursor for muscle protein synthesis in the continuous infusion protocol (as discussed by McNurlan and coworkers (1991)). McNurlan and coworkers (1991) refer to the study of Bennett and coworkers (1989) in which muscle protein synthesis rates are 2.3%/d when measured using muscle free leucine as precursor compared with 1.1%/d using plasma leucine and 1.3%/d using plasma alpha-ketoisocaproate. Therefore, using a continous infusion, depending on which precursor for protein synthesis is measured (for discussion see section 2.4), apparent muscle protein fractional synthetic rate may vary by as much as 100%. Finally, the fraction of the muscle biopsy that is hydrolysed for the determination of the incorporated amino acid enrichment (Halliday and McKeran, 1975) may also contribute to differences in the measured fractional synthetic rate (Table 7.9). That is,

the muscle preparation methods carried out on the muscle sample prior to hydrolysis can selectively alter the protein composition (thus the amino acid composition) of the sample (Forsberg et al., 1991). Measurements of muscle protein fractional synthetic rates in man have been carried out by measuring the incorporation of the labelled amino acid into different fractions of muscle tissue (Table 7.9). The fraction of muscle most commonly used in such studies is the alkali-soluble protein which is the cellular protein without the collagen protein of the muscle. In normal muscle, alkali-soluble protein has been reported to constitute 70% of the weight of the fat free solids and is only slightly reduced with age (Forsberg et al., 1991). However, it is not clear whether the proportion of alkali-soluble protein to the weight of fat free solids, in skeletal muscle, is further reduced in weight-losing cancer patients (Preston et al., 1987). Furthermore, it is not clear whether the muscle fraction used would alter the measured labelled leucine incorporation since collagen has little if any leucine residues. In contrast, the presence or absence of collagen from the muscle sample hydrolysed is likely to affect the enrichment of incorporated glycine since collagen is glycine-rich (33% of amino acid residues, see section 7.4.2). Therefore, in certain circumstances, the chosen tracer amino acid can potentially affect the measured fractional synthetic rate.

In summary, from the above discussion it is clear that a number of factors can affect the measured muscle protein fractional synthetic rate. However, it is not clear what the relative importance of each of these factors is in reported studies (Table 7.9) or in the present studies. The two reported studies (Emery et al., 1984a; Shaw et al., 1991) that have attempted to measure muscle protein fractional synthetic rates in weight-losing cancer patients, in vivo, report high values for their control group compared with normal subjects. Therefore, it would appear that the

value of these studies is in the comparative data obtained. The present study of muscle protein fractional synthetic rates in normal subjects has also yielded high values and probably should also be considered in this way.

There have only been two previous studies which have specifically attempted to compare in vivo muscle protein synthesis of normal subjects and weight-losing cancer patients. One has reported an increase in muscle protein fractional synthetic rate in cancer patients (Shaw et al., 1991) and the other a decrease (Emery et al., 1984). However, these studies differed in the plasma precursor used and the muscle fraction analysed for the enrichment of the bound amino acid. With reference to the factors discussed above which appear to affect the the measured fractional synthetic rate, the data presented in this thesis are more comparable with that of Shaw and coworkers (1991) and give a similar value for the muscle protein fractional synthetic rate in normal subjects (Table 7.9). Furthermore, the study of Shaw and coworkers (1991) reports an increase in the muscle protein fractional synthetic rate in weight-losing cancer patients (94%) which is in agreement with values obtained in the present studies (plasma glycine, 26%, homogenate free glycine, 93%, see Table 7.9). Finally, although different tracers were used, the increase in muscle protein synthesis is of sufficient magnitude to account for much of the apparent increase in whole body protein synthesis seen by both Shaw and coworkers (1991) and in the present study. Shaw and coworkers (1991) state "Because muscle is the largest protein reservoir in the body, it would take only a small increase in muscle protein synthesis to alter significantly whole body protein synthesis. In addition, the correlation between the fractional synthetic rate of protein in muscle and the rate of whole body protein synthesis supports this concept." However, in the

present study there was no correlation between the muscle protein fractional synthetic rate and the whole body protein synthesis rate when the values from both normal subjects and weight-losing cancer patients were considered. Furthermore, the measured protein synthetic rates are not consistent with the age, activity or nutritional status of the weight-losing cancer patients studied (Table 6.4). Also, in contrast to the conclusions of Shaw and coworkers (1991), there was no increase in skeletal muscle breakdown as measured by 3-methylhistidine excretion (Tables 5.9, 6.8). An increase in muscle protein synthesis without an accompanying increase in muscle protein breakdown would result in increased muscle mass. Since neither skeletal muscle mass (measured by creatinine excretion) nor muscle breakdown (measured by 3-methylhistidine excretion; Tables 5.9, 6.8) was increased this was clearly not the case in the patients studied in the present thesis.

The present work obviously does not preclude the possibility that important alterations in protein metabolism, sufficient to increase whole body protein synthesis occur in tissues other than muscle and liver. However, with specific reference to the increased incorporation of [^{15}N]glycine in the muscle of cancer patients, in the present studies, it is conceivable that certain glycine-rich proteins were present resulting in biased estimates of protein synthesis. When considering which protein or proteins might be involved in this process, there are several findings which should be considered. Firstly, that the fixed hepatic protein rates measured in the normal group are in accordance with those which might be expected, and that the synthesis rates were not increased in the cancer group, means it is unlikely that there was a systematic error (the presence of free [^{15}N]glycine) in the measurement of [^{15}N]glycine incorporation into liver and muscle tissues. Secondly, in order to increase whole body protein

kinetics the synthesis rate of the specific protein or proteins must be quantitatively significant. It follows that if such a protein or proteins were glycine-rich then this would proportionally enhance its effect on whole body and tissue specific synthesis rates measured by tracer glycine incorporation.

There are a limited number of glycine-rich proteins that could be involved in such a process. However, in patients with cancer cachexia the acute phase proteins (eg. fibrinogen, 10% glycine) and collagen (33% glycine) are of particular interest. The mechanism by which each protein could increase the [^{15}N]glycine content of the biopsy would be different. Fibrinogen, since it is synthesised by the liver and exported, could increase the [^{15}N]glycine enrichment of muscle tissue by contamination. Collagen, on the other hand, is synthesised in the muscle and therefore the increase in the [^{15}N]glycine enrichment would be a 'true' indicator of glycine incorporation into the biopsy sample but not an accurate indicator of skeletal muscle protein synthesis and may give misleading estimates of myofibrillar protein synthesis rates.

7.4.1 Increased muscle protein synthesis: Effect of acute phase protein synthesis

The subjects in the cancer group all had evidence of an acute phase protein response (ie. increased C-reactive protein, Table 7.1) and it is possible that the postulated proteins/protein which may have contaminated the muscle biopsy could have come from this group of hepatic export proteins. Of the proteins exported by the liver as part of the acute phase protein response, by far the most important quantitatively is fibrinogen (Table 7.8). Fibrinogen has a fundamental role in the clotting process since under attack by thrombin it undergoes a transformation into the long

thread-like polymer fibrin, the primary ingredient of a blood clot. The association between systemic activation of the clotting system and malignant disease has been recognised for more than a century (Sack, Levin and Bell, 1977; Dvorak, 1987). Patients with many types of malignancy, especially in their advanced stages, exhibit a diverse spectrum of coagulopathies that may include thrombophlebitis, haemorrhage, embolism and disseminated intravascular coagulation. As many as 98% of cancer patients (including those with colorectal cancer) may exhibit some type of clotting abnormality (Sun et al., 1979). Several authors have reported increased plasma fibrinogen concentrations in cancer patients (Sack, Levin and Bell, 1977) and the synthetic rate of fibrinogen is also increased (Stein et al., 1978a). Fibrinogen, in the normal individual, has the shortest half-life (approximately 85h; Stein et al., 1978b; Thompson et al., 1989) of the major plasma proteins. This occurs not only because fibrinogen is consumed in any clotting process, but also because the parent molecule seems to be particularly vulnerable to breakdown by proteolytic enzymes other than thrombin. Moreover, the degradation products resulting from such proteolysis may be involved in the stimulation of fibrinogen biosynthesis. Fibrinogen is not normally found outwith the vascular compartment because of its large molecular size (341,000daltons) but, in neoplastic disease fibrin is deposited both in solid tumours and normal tissues (Brown et al., 1988).

The mechanism by which fibrin is deposited in tissues has been investigated to some extent. Many tumours have been found to secrete a factor (vascular permeability factor) which increases blood vessel permeability such that the microvasculature about tumours becomes hyperpermeable to fibrinogen (Dvorak, 1986) and other plasma proteins (Fleck et al., 1985; Senger et al., 1983). This process also occurs in

normal tissues, but to a lesser extent. Extravasated fibrinogen is converted rapidly to fibrin by means of procoagulants associated with tumour and perhaps with other tissues (Dvorak et al., 1985). This increased volume of distribution together with increased fibrinogen concentration in the plasma suggests there may be a substantial increase in synthesis of this glycine-rich protein.

In the current studies (Chapters 5 and 6) fibrinogen would not have been removed from the muscle samples by the acid/alcohol wash as part of the sample preparation procedure since much lower concentrations of neutral pH alcohol would be required to ensure solubilisation (about 9% ethanol). Furthermore, fibrin or fibrinogen degradative products formed from fibrinogen would not have been removed from the muscle. The formation of fibrin and together with the insoluble nature of such material in the tumour and normal tissues of the cancer host (eg. muscle) has been shown by Brown and coworkers (1988). These investigators demonstrated clearly the influx of ^{125}I-labelled fibrinogen into the tumour and normal tissues of tumour-bearing mice. The influx into the muscle was approximately one third that of the flux into the transplanted carcinoma. Given the larger mass of muscle in man it is concievable that this mechanism of fibrinogen deposition represents a significant removal and trapping of free glycine of cancer patients with an acute phase response. Confirmatory evidence for this mechanism would require demonstration of fibrin deposits in the muscle of the cancer group and demonstration of a significantly increased fibrinogen synthetic rate. However, from the literature such evidence is inconclusive. This lack of evidence could be due to the fact that methods to identify fibrin are complicated and technically difficult. The methodology to assess fibrin deposition or fibrinogen synthetic rates were not developed in the present study, however,

increased fibrinolysis was demonstrated in the cancer group when compared to the control group (Table 7.1).

If we assume that total liver protein synthesis can account for approximately 20% of whole body protein synthesis (Waterlow, Garlick and Millward, 1978d; Shaw et al., 1991) and that half of total liver protein synthesis is export protein synthesis then this would account for approximately 22gP/d in the control group and 35gP/d in the cancer group (Table 7.3). Therefore, from the above calculation, even if all liver export protein was fibrinogen, it could not explain the entire increase in [^{15}N]glycine muscle incorporation of the cancer group (Table 7.3). Nevertheless, fibrin/fibrinogen deposition in normal tissues (see above) such as muscle may contribute to the increased [^{15}N]glycine enrichment in the muscle of the cancer group.

An increase in the fractional synthetic rate of fibrinogen in cancer patients has been reported. It has been reported to be 15%/d in normal subjects (Stein et al., 1978a) and 26%/d in cancer patients (Stein et al., 1978b). However, the degree of inflammatory response in the cancer patients is not clear from the report by Stein and coworkers (1978b) and it is conceivable that the cancer patients in our study (Chapter 6), with a significant elaboration of acute phase proteins (Table 7.1), had an even greater increase in fibrinogen turnover. It has been reported that in burns patients fibrinogen synthesis can be increased to 85%/d (Thompson et al., 1989) and if this were the case in the patients in the present study this suggests that fibrinogen could indeed be responsible for some of the increased [^{15}N]glycine enrichment of the skeletal muscle biopsies in the cancer group.

In summary, fibrinogen synthesis is likely to have been increased in the cancer group and could potentially, through contamination, be

responsible for a significant proportion of the apparent increase in skeletal muscle protein synthesis (i.e. [^{15}N]glycine enrichment).

7.4.2 Increased muscle protein synthesis: Effect of collagen synthesis

Another protein which might contribute to an apparent increase in muscle protein synthesis would be collagen. It is the most abundant protein in mammals, accounting for approximately a quarter of total body protein. It is present in nearly all organs and serves to hold cells together in discrete units. In the mouse for example, collagen represents 20% of total protein (of which 40% is in the skin, and 50% in the bone and muscle; Waterlow, Garlick and Millward, 1978h). The proportion of glycine residues in collagen is approximately 33% which is unusually high for a protein (e.g glycine content of haemoglobin is 5% (Stryer, 1988). Therefore, collagen accounts for approximately half of the body's bound glycine.

Collagen is synthesised in fibroblasts on the membrane-bound ribosomes of the rough endoplasmic reticulum and is a secreted protein. In common with most export proteins collagen is synthesised as a precursor molecule, procollagen, some 15-30% larger than collagen. The formation of the collagen fibre is analogous to the formation of fibrin fibres. Indeed, fibrin is replaced by collagen when a clot becomes organised.

Collagen accounts for 8.75gP/kg of 'normal' muscle (Geigy Scientific Tables) and therefore, there is 154g muscle collagen/70kg adult (assuming 17.6kg muscle/70kg adult). Since glycine constitutes 33% of the amino acid residues of collagen then using glycine incorporation to measure muscle protein synthesis (where the protein was collagen) would give a value of 847gP (that is 5.5 times the mixed protein value). Thus, to account for the increase in protein turnover in skeletal muscle measured by

glycine flux (125gP/d), assuming no compositional change, then the fractional synthetic rate of collagen in muscle would have to be approximately 14%/d. Total collagen synthesis in the body is thought to be about 1%/d (Waterlow, Garlick and Millward, 1978h) but there is likely to be a difference between bone, which accounts for up to 50% of body collagen, and tissue collagen. The part played by tissue collagen is likely to be quantitatively important since in malnutrition the amount of collagen in the body is relatively increased, and that of cellular protein decreased. This has been shown in the pig, cockerel (Dickerson and McCance, 1964) and man (Picou, Halliday and Garrow, 1966). Therefore, collagen does not share in the general depletion of body protein. Animal experiments have shown that the amount of collagen is more stable in some tissues than others. In rats, cockerels, and pigs on a low protein diet the collagen content of muscle was found to be increased; the muscle mass was greatly reduced, but the absolute amount of collagen in an individual muscle was the same as in animals on a normal diet (Mendes and Waterlow, 1958; Dickerson and McCance, 1964). On a low protein diet it has been shown, with photomicrographs, that muscle tissue shrinks inside its collagen scaffolding, (Montgomery, 1962). This shrinking or atrophy of skeletal muscle fibres has also been demonstrated, using quantitative histological and histochemical techniques, in weight-losing cancer patients (Lindboe and Torvik, 1982)

The turnover of collagen is thought to be necessary for structural modifications of the body which accompany growth or any condition which alters body structure. In adult man about half of the total collagen is present in bone and therefore in the normal subject hydroxyproline excretion is considered to be a reflection of bone collagen turnover (Dull and Henneman, 1963; Guzzo and Kivirikko, 1967). However, attempts to

use hydroxyproline as a marker for bone disease have met with mixed success. It appears that unless there is significant bone breakdown, in the absence of any other pathology, hydroxyproline excretion data is of limited value. Increased hydroxyproline excretion is found in most cancer patients with or without bone metastases. Without bone metastases the urinary hydroxyproline concentration increases 2-3 times, with bone metastases it increases 4-10 times that of the normal subject (Guzzo et al., 1969). Therefore, in the cancer patients examined in this study who had no evidence of bone metastases, measurement of urinary hydroxyproline might give an indication of increased collagen turnover. From the results (Table 7.5) this would appear not to be the case since there was no increase in the urinary hydroxyproline excretion when compared with the control group.

Nevertheless, the presence of collagen in the muscle fraction analysed for [^{15}N]glycine enrichment may contribute to higher muscle protein fractional synthetic rates in the control group compared to other studies (see section 7.4). It is of interest that 3 subjects in the cancer group had detectable serum concentrations of tumour necrosis factor (see Table 7.4). Tumour necrosis factor is known to play a major role in tissue inflammation and remodelling by stimulating production of tissue collagenases (Evans, Argiles and Williamson, 1989; Vlassara et al., 1988). Therefore, it is possible that, in the cancer group, increased muscle collagen synthesis may contribute to the apparently increased muscle protein fractional synthetic rate.

7.4.3 Increased muscle protein synthesis: summary

In summary, to explain the majority of increased [^{15}N]glycine incorporation into the muscle of the cancer patient it is proposed that the

amounts of glycine-rich proteins, such as fibrinogen, in muscle is increased. Collagen, as a major glycine-rich structural protein, was also considered likely to be involved in such a proposed mechanism since the relative amount of muscle collagen may increase as the cachexia develops in the cancer patient. However, in the present study (Chapters 5 and 6) there was little evidence of changes in the muscle metabolism of the cancer patients (no significant difference in urinary creatinine or hydroxyproline excretion) to support the involvement of collagen in the above process.

7.5 Apparently elevated whole body protein synthesis rates in weight-losing cancer patients: A hypothesis

Very lame and imperfect theories are sufficient to suggest useful experiments which serve to correct those theories and give birth to others more perfect. These, then, occasion further experiments which bring us still nearer to the truth; and in this method of approximation we must be content to proceed, and we ought to think ourselves happy if, in this slow method, we make any real progress.

Joseph Priestly

(quoted by Waterlow, Garlick and Millward, 1978)

As has been discussed already (Chapter 2) the calculation of protein synthesis from glycine flux requires a model of protein metabolism which is based on a number of assumptions. The concept of a single pool of nitrogen in the body is perhaps the most fundamental assumption of the 'end-product' methods that have been used for measuring the rate of whole body protein metabolism in man. It is assumed that the metabolically active nitrogen (and tracer ^{15}N) in the body is contained in a single

homogenous pool into which nitrogen from the diet and from protein breakdown enters, and from which nitrogen is removed for the synthesis of proteins and excretory products. This concept, first proposed by Springson and Rittenberg (1949), therefore assumes that there is uniform distribution of nitrogen throughout the body and that there is unrestricted metabolic exchange between the free amino acids of this pool. In reality, the situation may be much more complex with more than one free amino nitrogen pool, that is, compartmentation of the precursor pool for protein synthesis. This has been demonstrated in rats (Fern and Garlick, 1976) and proposed to occur in normal man (Fern et al., 1985a, 1985b) when using [^{14}C] and [^{15}N]glycine respectively. Nevertheless, the idea of a single precursor pool for protein synthesis has been widely accepted in practice, and has remained a central feature of many subsequent models and studies of whole-body nitrogen metabolism in man and in animals. This acceptance stems mainly from an absence of workable alternative models and appears to give reasonable comparative results in normal man (Garlick and Fern, 1985).

A hypothesis for the aetiology of the compartmentation of amino acid metabolism has been put forward by Stein and coworkers (1983). He proposes that the normal response to metabolic stress (alterations in substrate metabolism resulting from injury, infection, cancer) is an increase in protein turnover which imposes additional demands on the liver. If the liver's 'metabolic capacity' is exceeded, it no longer has the capacity to supply substrates, process waste products and interconvert metabolites which have been partially metabolised by one tissue and are necessary for normal peripheral metabolism. The impairment of liver function will thus affect amino acid metabolism as there will no longer be unimpeded transfer and exchange between the various tissue pools of amino acids. Therefore,

under these circumstances, the single amino acid pool of the model may not be valid and the measurement of whole body protein turnover could be biased. In Chapter 6, it was demonstrated that structural hepatic protein synthesis in the cancer group was reduced significantly and may therefore further compromise the ability of these patients to deal with the metabolic stress of cancer. Stein's evidence to link impaired liver function with the lack of validity of the current model was simply that there was a good correlation between elevated whole body protein synthesis and abnormal liver function tests in the metabolically stressed patient.

Further evidence of the relationship between the degree of metabolic stress (in patients with cancer) and whole body protein turnover measurements has been demonstrated by a number of workers using [^{15}N]glycine (Stein et al., 1983; Fearon et al., 1990). In particular, Carmichael and coworkers (1980) using [^{14}C]leucine reported a correlation between tumour burden as defined by tumour mass/anatomical staging and whole body protein synthesis rates. These findings have been confirmed using [^{15}N]glycine (Fearon et al., 1988; Fearon et al., 1990). Furthermore, it has been shown that the degree to which protein turnover is elevated relates to the ultimate duration of survival of patients with colorectal cancer (Fearon et al., 1990).

Thus, it would appear that irrespective of whether leucine or glycine is used to measure whole body protein turnover there is a relationship between whole body protein turnover and the degree of metabolic stress imposed by cancer. However, such studies certainly do not establish a cause and effect relationship (as proposed by Stein and coworkers, 1983) between metabolic stress causing liver dysfunction and the observed changes in tracer amino acid flux.

With reference to that discussed previously (see 7.4.1), an alternative hypothesis, which I propose to account for the apparently elevated whole body protein turnover rates of cancer patients, would be that the measurement is influenced by an inflammatory response and the subsequent enhanced turnover of fibrinogen and other glycine-rich proteins (see section 7.4.1, 7.4.2, 7.6) results in a significant proportion of the total glycine pool being diverted for their synthesis. Such enhanced fibrinogen synthesis would result in [^{15}N]glycine being removed and not being returned to the circulation during the course of the experiment. This hypothesis would also explain why measurements (using [^{15}N]glycine) made on patients who were fed (Glass, Fern and Garlick, 1983) showed no significant increase in whole body protein turnover, since the glycine pool would have been expanded and the body would be more able to supply the demand for glycine. Therefore, glycine flux would be less biased towards the synthesis of glycine-rich proteins (Grimble, 1990). Furthermore, the increased synthesis of these glycine-rich proteins as part of the inflammatory response in cancer patients may explain why the increase in whole body protein turnover measured in similar control and cancer groups is greater with [^{15}N]glycine (Fearon et al., 1988) compared with that obtained with [^{13}C]leucine (Inculet et al., 1987; Melville et al., 1989; see Table 7.2).

7.6 The mediators of increased fibrinogen synthesis in weight-losing cancer patients

In the previous sections of this Chapter it has been proposed that the increase in skeletal muscle protein synthesis, measured using [^{15}N]glycine, in the cancer patients might be explained by the presence of glycine-rich proteins, in particular, fibrinogen and perhaps by the increased

synthesis of collagen in the muscle (although there was no evidence for the latter process in the cancer group). Furthermore, it would appear that increased glycine-rich protein synthesis in the cancer group may have a significant influence on the body glycine pool, possibly introducing a bias in the whole body protein synthesis value estimated using [^{15}N]glycine. However, is not clear what the metabolic basis of such increased glycine-rich protein synthesis might be.

Increased fibrinogen synthesis might be part of a generalised inflammatory response in the cancer patients (as documented by an increased plasma concentration of C-reactive protein (Table 7.1) (Pepys and Baltz, 1983)). This is in accord with other published work which suggests that the majority of cancer patients with progressive disease will develop such a inflammatory response elaborating acute phase proteins (Raynes and Cooper, 1983; Cooper and Stone, 1979; Fearon and Carter, 1988). The question which next arises is what might be the mediators of such an acute phase response? From the literature there appear to be two main mediators of increased fibrinogen synthesis, namely cortisol and interleukin-6. The role of these factors in cancer patients' metabolic abnormalities will be discussed in the following section.

7.6.1 The influence of cortisol and Interleukin-6 on fibrinogen synthesis

It is well established that increasing glucocorticoid (cortisol) concentration in blood results in increased fibrinogen synthesis (Waterlow, Garlick and Millward, 1978g). Furthermore, there is mounting evidence that, as part of the general inflammatory response, the cytokine interleukin-6 is a primary mediator of the increase in synthesis of acute phase proteins, including fibrinogen, (Heinrich, Castell and Andus, 1990). Other cytokines such as interleukin-1 and tumour necrosis factor alpha also

stimulate fibroblasts, endothelial cells and keratinocytes to synthesise interleukin-6 and thus amplify its biological effects. Moreover, interleukin-6, interleukin-1 and tumour necrosis factor alpha have been shown to stimulate the release of adrenocorticotrophic hormone from pituitary cells, leading to an increased secretion of glucocorticoids including cortisol. The glucocorticoids can increase the stimulatory effect of cytokines on the synthesis of acute phase proteins by hepatocytes and also inhibit the synthesis of the monokines. With particular reference to fibrinogen, interleukin-6 is the only cytokine that has been shown to increase synthesis in man (Heinrich, Castell and Andus, 1990). Furthermore, it has been demonstrated that in vitro in the rat hepatoma cell line recombinant human interleukin-6 can produce a six fold increase in fibrinogen mRNA (Andus et al., 1988).

Therefore, in the present study both cortisol and interleukin-6 were measured on the basal serum and plasma samples of the cancer patients and controls (see protocol Chapter 5). From the results (Table 7.6) it can be seen that there was a significant increase in both the cortisol and interleukin-6 concentrations in the cancer group compared with the controls. This was in the presence of low insulin concentrations (many of the metabolic effects of cortisol are dependent on insulin concentration being low at the same time; Johnston et al., 1982) and normal glucose concentrations, with the exception of subject 5 in the cancer group (Table 7.7). A similar elevated cortisol:insulin ratio has been reported in weight-losing cancer patients by Selberg and coworkers (1990). Thus, with the established effects of interleukin-6 and cortisol, the raised concentrations of these mediators could have been responsible for the elevated fibrinogen synthesis. Furthermore, in the present study there was a statistically significant correlation between the interleukin-6 and

fibrinogen concentrations ($r^2 = 58.5\%$, $p = 0.01$, $n = 10$) but, not with cortisol and fibrinogen ($r^2 = 11\%$, $p = 0.291$, $n = 12$). However, when the concentration of cortisol was multiplied by that of interleukin-6 and the resultant product correlated with that of fibrinogen the relationship became even more significant ($r^2 = 66\%$, $p = 0.004$, $n = 10$). In addition, the most significant correlation was between the product of cortisol multiplied by interleukin-6 and fibrin degradative product values ($r^2 = 82.8$, $p < 0.001$, $n = 10$). In summary, these data are consistent with increased fibrinogen turnover, as part of the acute phase protein response, mediated by cortisol and interleukin-6.

7.6.2 Comparison with other work

Clague and coworkers (1982) have previously described an increase in whole body [^{14}C]leucine flux associated with increased incorporation of [^{14}C]leucine into plasma proteins. In patients with colorectal cancer there was an increase in the percentage of the total dose administered that was incorporated into plasma proteins with increasing advancement of cancer. The mean percentages were; Duke's A, 6.7%; Duke's B, 8.3%; Duke's C, 9.4%, Dukes D, 11.8%. In these calculations the authors assumed that the distribution of these proteins was confined to the plasma volume. However, with reference to the more advanced cases of colorectal cancer, the volume of distribution is likely to be much greater (discussed in 7.4.1) and therefore the percentage of the total dose incorporated into the plasma proteins is likely to be substantially underestimated in the advanced cancer patients. Clague and coworkers (1982) also reported a statistically significant correlation between the percentage increase in plasma cortisol levels postoperatively and the increase in the percentage incorporation of [^{14}C]leucine into plasma proteins ($r = 0.84$, $p < 0.01$) in a group of patients

undergoing elective cholecystectomy. In order to find out which of the plasma proteins were responsible for this increase, the plasma was fractionated by gel filtration and the eluted fractions counted. The radioactivity was then plotted against the eluted fraction for each plasma sample to assess the distribution of counts throughout the various plasma proteins. Examples, were given of the different profiles, but no attempt was made to adjust the [^{14}C]leucine activity to the amino acid composition of the protein. That is, different proteins contain different amounts of leucine (% of amino acid residues), for example, albumin is 8.9% leucine whereas fibrinogen is 6.3%. In contrast the amino acid composition of human albumin is 3% glycine and fibrinogen is 9.9% (Grimble, 1990 ; Cartwright and Kekwick, 1970).

If this were done then the the radioactivity profile would then give some idea of the relative amounts of protein that were being synthesised. Taking the example of albumin and fibrinogen it would appear that relatively more fibrinogen was being synthesised (29%). Another point of interest from these plasma protein incorporation profiles was that of a unknown peak of activity with a molecular weight less than albumin (66,000daltons). Clague and coworkers (1982) do not suggest what this protein might be. From the results presented in this Chapter, one suggestion for this protein fraction would be fibrinogen/fibrin degradative products which is the term covering the peptide fragments with molecular weights ranging from approximately 10,000-50,000daltons. The concentration of degradation products from cross linked fibrin were measured and found to be increased in the cancer group in the present study (Table 7.1).

7.7 Summary

The alteration in protein synthesis in weight-losing cancer patients may be biased towards the increased synthesis of glycine-rich proteins, probably as part of the inflammatory response to the tumour. Thus, with reference to the model for the calculation of whole body protein synthesis, the assumption that similar mixes of protein are being synthesised in the cancer and control subjects is possibly invalid resulting in an overestimate of whole body protein synthesis when [^{15}N]glycine is used. Furthermore, the deposition of glycine-rich structural proteins such as fibrinogen and/or increased collagen synthesis in muscle tissue might account for increased glycine enrichment of skeletal muscle independent of any alteration in the synthesis of cellular proteins in the tissue itself. However, confirmation of this hypothesis will require a demonstration of increased fibrinogen synthesis and/or fibrin deposition and/or collagen synthesis in the tissues of patients with cancer and altered protein kinetics.

Table 7.1

Changes in plasma protein concentrations in normal subjects and weight-losing cancer patients

		C-reactive protein (mg/l)	Fibrinogen (g/l)	Albumin (g/l)	FDP (ug/l)
Control	1	10	3.3	40	207
	2	10	2.5	40	141
	3	6	4.5	39	388
	4	10	4.1	43	184
	5	8	3.0	41	126
	6	8	4.2	41	243
Mean		9	3.6	41	215
S.D.		2	0.8	1	95
Cancer	1	120	6.7	34	1363
	2	41	4.6	24	394
	3	11	5.5	39	523
	4	115	6.8	35	1310
	5	52	5.5	39	201
	6	108	4.9	31	1092
Mean		75*	5.7**	34**	814*
S.D.		46	0.9	6	502

FDP, fibrin degradative products, * $p<0.05$, ** $P<0.01$

Table 7.2

Comparison of whole body protein turnover in normal subjects and cancer patients as determined by different investigators

Author (ref)	Tracer	Protein turnover (g protein/kg/d)			
		weight-stable controls	weight-losing controls	weight-stable patients	weight-losing patients
Heber (a)	[^{14}C]lys	1.9			3.2(68)
Inculet (b)	[^{13}C]leu	3.9		4.5(15)	
Jeevanandam (c)	[^{15}N]gly^{x}		2.4		3.2(33)
Norton (d)	[^{15}N]gly^{y}	2.9			5.0(72)
Kien (e)	[^{15}N]gly^{y}	3.5			5.5(57)
Ward (f)	[^{15}N]gly^{z}	2.9		2.7(-7)	3.9(34)
Fearon (g)	[^{15}N]gly^{z}	3.1	4.6(48)	4.6(48)	4.9(58)
Fearon (g)	[^{15}N]gly^{y}	2.2	3.5(59)	3.6(64)	4.0(82)
Melville (h)	[^{13}C]leu	3.1		3.7(19)	
This work	[^{15}N]gly^{y}	3.5			5.9(69)

Mean value (% increase from control)
(a) Heber et al., 1982 (b) Inculet et al., 1987 (c) Jeevanandam et al., 1984 (d) Norton, Stein and Brennan, 1981 (e) Kien and Camitta, 1983 (f) Ward et al., 1985 (g) Fearon et al., 1988 (h) Melville et al., 1989.
^{x} end-product average, ^{y} ammonia end-product, ^{z} urea end-product (see section 2.3).

Table 7.3

Whole body, liver and skeletal muscle protein synthesis in normal subjects and weight-losing cancer patients

	Protein synthesis (gP/d)			
	WBPS[a]	MPS[b]	LPS[a]	LPS+MPS/WBPS[b] (%)
Control	217(31)	96(15)	22(1)	61(14)
Cancer	345(35)*	214(76)*	15(2)**	67(10)

All values, Mean(SEM), in grams Protein/day, [a] n=6, [b] n=4, *p<0.05, **p<0.01

Table 7.4

Rates of protein synthesis and potential mediators in normal subjects and weight-losing cancer patients

		TNF[a] (pg/ml)	Hydroxyproline /Creatinine	MPS (gP/kg/d)	WBPS
Control	1	<12	0.085	0.9	3.3
	2	<12	0.032	1.4	2.4
	3	<12	0.076	1.1	5.1
	4	<12	0.130		2.6
	5	<12	0.059	1.8	2.0
	6	<12	0.018	1.7	3.4
Mean			0.066	1.4	3.1
S.D.			0.040	0.4	1.1
Cancer	1	15	0.091	2.7	5.0
	2	22	0.111	3.0	7.3
	3	<12	0.035	2.6	2.8
	4	<12	0.018		5.0
	5	<12	0.095	4.2	5.3
	6	15	0.034	3.2	6.5
Mean			0.064	3.1*	5.3*
S.D.			0.039	0.6	1.5

[a] Method described in Fearon et al., 1991, TNF tumour necrosis factor, MPS, muscle protein synthesis; WBPS, whole body protein synthesis; * $p<0.05$, ** $P<0.01$

Table 7.5

Urinary hydroxyproline excretion in normal subjects and weight-losing cancer patients

		Hydroxyproline (mmol/vol)	Creatinine (mmol/vol)	Hydroxyproline /Creatinine
Control	1	0.290	3.4	0.085
	2	0.172	5.4	0.032
	3	0.266	3.5	0.076
	4	0.468	3.6	0.130
	5	0.161	2.7	0.059
	6	0.155	8.5	0.018
Mean		0.252	4.5	0.066
S.D.		0.120	2.1	0.040
Cancer	1	0.312	3.4	0.091
	2	0.100	0.9	0.111
	3	0.247	6.9	0.035
	4	0.139	7.7	0.018
	5	0.498	5.2	0.095
	6	0.372	10.7	0.034
Mean		0.278	5.8	0.064
S.D.		0.149	3.4	0.039

Hydroxyproline/Creatinine reference range 0.003-0.033

Table 7.6

Rates of protein synthesis and potential mediators in normal subjects and weight-losing cancer patients

		Cortisol (nmol/l)	Il-6 (u/ml)	CRP (mg/l)	Fibrinogen (g/l)	MPS (gP/kg/d)	WBPS
Control	1	324	16	10	3.3	0.9	3.3
	2	542	10	10	2.5	1.4	2.4
	3	164	<10	6	4.5	1.1	5.1
	4	201	<10	10	4.1		2.6
	5	192	14	8	3.0	1.8	2.0
	6	307	13	8	4.2	1.7	3.4
Mean		288		9	3.6	1.4	3.1
S.D.		140		2	0.8	0.4	1.1
Cancer	1	399	330	120	6.7	2.7	5.0
	2	452	96	41	4.6	3.0	7.3
	3	230	26	11	5.5	2.6	2.8
	4	608	170	115	6.8		5.0
	5	575	70	52	5.5	4.2	5.3
	6	543	118	108	4.9	3.2	6.5
Mean		468*	135	75*	5.7**	3.1*	5.3*
S.D.		140	107	46	0.9	0.6	1.5

MPS, muscle protein synthesis; WBPS, whole body protein synthesis; * $p<0.05$, ** $P<0.01$

Table 7.7

Fasting plasma insulin and glucose concentrations in normal subjects and weight-losing cancer patients

		Insulin (mU/l)	Glucose (mmol/l)
Control	1	18.1	5.4
	2	12.6	4.4
	3	9.1	4.8
	4	13.4	4.6
	5	<3.0	4.7
	6	<3.0	4.5
Mean			4.7
S.D.			0.4
Cancer	1	<3.0	3.5
	2	<3.0	5.9
	3	10.8	4.5
	4	17.7	6.7
	5	48.0	10.8
	6	<3.0	4.6
Mean			6.0
S.E.M.			2.6

Table 7.8

The acute phase plasma proteins in man

	Mol.wt (d)	Concn (g/l)	Carb.cont. (%)	Glycine (% aa residues)	Serine
Fibrinogen	341,000	3.0	2.5	10	8
Haptoglobin	99,000	2.0	19.3	7	5
Alpha-1-Antitrypsin	54,000	2.0	12.4	5	5
Alpha-1-Acid glycoprotein	44,000	1.0	41.4	4	4
Haemopexin	57,000	0.8	22.6	-	-
Caeruloplasmin	130,000	0.2	8.0	7	6
C-reactive protein	105,000	<0.01	0	8	10
Serum Amyloid A	12,000	<0.001	0	12	9

Mol.wt= Molecular weight, (d)= daltons, Concn= Normal plasma concentration, Carb.cont.= Carbohydrate content, % aa residues= % of amino acid residues. From Fleck, Colley and Myers, 1985; Grimble, 1990; Cartwright and Kekwick, 1971.

Table 7.9

Comparison of skeletal muscle protein synthesis in vivo in normal subjects and cancer patients as determined by different investigators

Author (ref)	Precursor used	Protein FSR (%/d) weight-stable subjects	Protein FSR (%/d) weight-losing patients	muscle fraction
Halliday (a)	plasma lys	3.8(0.4)		sarcoplas
		1.4(0.3)		myofibril
Rennie (b)	plasma KIC	2.4(0.4)		ASP
Halliday (c)	plasma KIC	1.1(0.1)		ASP
Emery (d)	plasma KIC	4.8(0.5)	0.7(0.2)	ASP
Garlick (e)	plasma KIC	1.9(0.1)		ASP
Shaw (f)	plasma leu	2.4(0.4)	4.6(0.9)	FFS
This work	plasma gly	2.3(0.4)	2.9(0.2)	solids
	homog gly	2.8(0.3)	5.4(0.5)	solids

Mean value (SEM)
(a) Halliday and Mckeran, 1975 (b) Rennie et al., 1982 (c) Halliday et al., 1988 (d) Emery et al., 1984a (e) Garlick et al., 1989 (f) Shaw et al., 1991.
lys, free lysine; KIC, free alphaketoisocaproate; leu, free leucine; gly, free glycine; homog, homogenate.
sarcoplas, sarcoplasmic; myofibril, myofibrillar; ASP, alkali-soluble protein; FFS, fat-free solids.

CHAPTER 8: CONCLUSION

8.1 Introduction

In chapter 1 the aims of this thesis were defined as follows;

1. To develop methods whereby whole body and tissue protein fractional synthetic rates could be measured using a single mass spectrometer.
2. To establish a clinical protocol to measure simultaneously resting energy expenditure and whole body, fixed hepatic and skeletal muscle protein synthesis in man.
3. To use this protocol to compare the protein and energy metabolism in normal subjects with those in weight-losing cancer patients.

8.2 Aim 1

There have been several attempts over the years to establish a single methodology for the determination of the product and precursor pool specific activity/enrichment. One reason for this is that the potential for systematic analytical errors introduced by the use of different methods is minimised and thus the precision and accuracy of data derived from such studies may be increased. Attempts to use Gas Chromatography-Mass Spectrometry to measure both free and bound amino acid isotopic enrichment have required the use of a non-tracer dose of the labelled amino acid which may invalidate the synthetic rate measurements (see Chapter 2). Horber and coworkers (1989) have described the separation of labelled leucine from plasma and hydrolysed tissues, prior to the measurement of specific radioactivity, using an isocratic high performance liquid chromatographic system. They reported that fractional protein synthetic rates in a variety of tissues could be determined with better

accuracy and precision, and that the analysis time was similar to that required using conventional radioactive isotope methodology. However, the use of radioactive isotopes for tracer studies in man is largely precluded on ethical grounds. Halliday and McKeran (1975) used liquid chromatography for the separation of the stable isotope labelled amino acid lysine from plasma and tissue hydrolysate samples prior to measurement by Isotope Ratio Mass Spectrometry. However, the method was slow and required large amounts of plasma for isotopic analysis. Considering the first aim it has been demonstrated in this thesis that Isotope Ratio Mass Spectrometry in the form of Continuous Flow-Isotope Ratio Mass Spectrometry can be used in conjunction with high performance liquid chromatography to measure the isotopic enrichment of both plasma/tissue free and protein bound glycine using a tracer dose of the amino acid (Chapter 4.2-4.6). Therefore Continuous Flow-Isotope Ratio Mass Spectrometry together with high performance liquid chromatography offers an alternative to the conventional instrumentation (Gas Chromatography-Mass Spectrometry and Isotope Ratio Mass Spectrometry) for stable isotope tracer work and does not introduce the complication of cross calibrating different mass spectrometers. The work presented in this thesis is only the second report of liquid chromatography in conjunction with isotope ratio mass spectrometry being used to measure fractional protein synthetic rates in man. Furthermore, it appears from the literature that it is the first to report the use of a volatile buffer, with consequent advantages in sample preparation, in the separation of amino acids in tracer studies.

8.3 Aim 2

In normal individuals, measurement of whole body and tissue specific amino acid kinetics was achieved with the use of [^{15}N]glycine but

not [^{13}C]leucine (Chapter 5). The use of leucine was restricted by the limitations of the instrumentation used for measuring tissue homogenate free [^{13}C]leucine enrichment. In the control subjects the clinical protocol gave similar resting energy expenditure and whole body protein turnover values to other studies and a plasma plateau of [^{15}N]glycine enrichment was established over the time of the infusion. With reference to data in the literature it would appear that the values obtained for non-export liver protein synthesis in the control group were reasonable, being only the second report in man. However, values for muscle protein synthesis were above published values measuring [^{13}C]leucine incorporation in alkali soluble protein, but in accord with values recently published measuring [^{14}C]leucine incorporation in fat free solids (Table 7.9).

8.4 Aim 3

Whole body and tissue specific rates of protein synthesis were measured in a group of weight-losing cancer using the same [^{15}N]glycine protocol as that used for the controls (see Aim 2). Whole body amino acid kinetics were found to be grossly elevated but this was not associated with an increase in resting energy expenditure (Chapter 6). Labelled glycine incorporation into hepatic fixed protein was significantly reduced whilst tracer incorporation into skeletal muscle appeared to be significantly increased (Chapter 6). However, critical assessment of these results appears to draw attention to the limitations of extrapolating the kinetics of a single labelled amino acid to the rate of synthesis of protein either in individual tissues or in the whole body (Chapter 7). In particular, from the work presented in this thesis and the work of Emery and coworkers (1984a) and Shaw and coworkers (1991), it would appear that depending on the tracer amino acid used, muscle protein synthesis may be either

increased or decreased in the cancer host. One reason for such a divergence in the apparent protein synthesis rates might be that there is an alteration in the pattern of proteins synthesised/or found in the muscle of cancer patients. This would result in amino acids being incorporated into proteins at different rates from that in normal subjects. This may explain why the documented increase in whole body protein synthesis in cancer patients appears to be greater when [^{15}N]glycine is used compared with [^{13}C]leucine (Table 7.2). Therefore, in order to obtain values which more truely reflect protein synthesis in the whole body and different tissues in weight-losing cancer patients it would seem necessary to use more than one labelled amino acid and integrate the kinetic data with that obtained from the conventional nitrogen balance technique (Bier, Matthews and Young, 1985). Indeed, this approach has been used in small infants and gives lower and more plausible results than with a single tracer (Heine, 1988). The protein synthetic rate of the tissue could then be calculated by adjusting the individual amino acid flux measurements to the amino acid composition of the tissue protein and then averaging the corrected amino acid flux rates.

Thus, despite the fact that at least ten studies over the last decade have shown that whole body "protein turnover" is increased in cancer patients (with and without weight-loss) (Table 7.2) such results should be treated with caution since these measurements have been made using single amino acid tracers and there has been no concomitant increase in resting energy expenditure. With regard to measurement of muscle protein synthesis, it is possible that in weight-losing cancer patients the protein composition (and therefore the amino acid uptake) of muscle changes markedly and this must be considered when interpretating tracer incorporation data. It must be noted, however, that despite uncertainty

about the true significance of the altered tracer kinetics documented in the present study, these results emphasise the markedly altered metabolism of the weight-losing cancer host (Figure 8.1).

How such changes in host protein metabolism are mediated remains an open question. It is still not clear whether such abnormalities are induced by a factor or factors produced by the tumour itself or by the host in response to the tumour. Nevertheless, in the present study, hepatic fixed protein synthesis was shown to be decreased (Table 6.3) whilst the plasma concentration of at least two acute phase hepatic export proteins was increased (fibrinogen and C-reactive protein, Table 7.1). This reprioritization of hepatic protein synthesis (Sganga et al., 1985) was associated with significant elevation of serum cortisol and interleukin-6 concentrations (Table 7.6). Both these factors are known to influence hepatic acute phase protein production and may well have been responsible for at least some of the changes in hepatic protein metabolism. Furthermore, if as suggested (Chapter 7.4.1) increased production of glycine-rich hepatic export proteins such as fibrinogen may bias whole body and tissue specific protein kinetics measured by [^{15}N]glycine then cortisol and interleukin-6 may also influence, albeit indirectly, some of the extra-hepatic changes in cancer patients' protein metabolism. Further studies will be required to define the altered protein metabolism and mediators of such changes in cancer patients. However, it is to be hoped that by elucidating the mechanisms of weight loss in cancer, better methods of supporting and improving the nutritional and metabolic status of such patients will result.

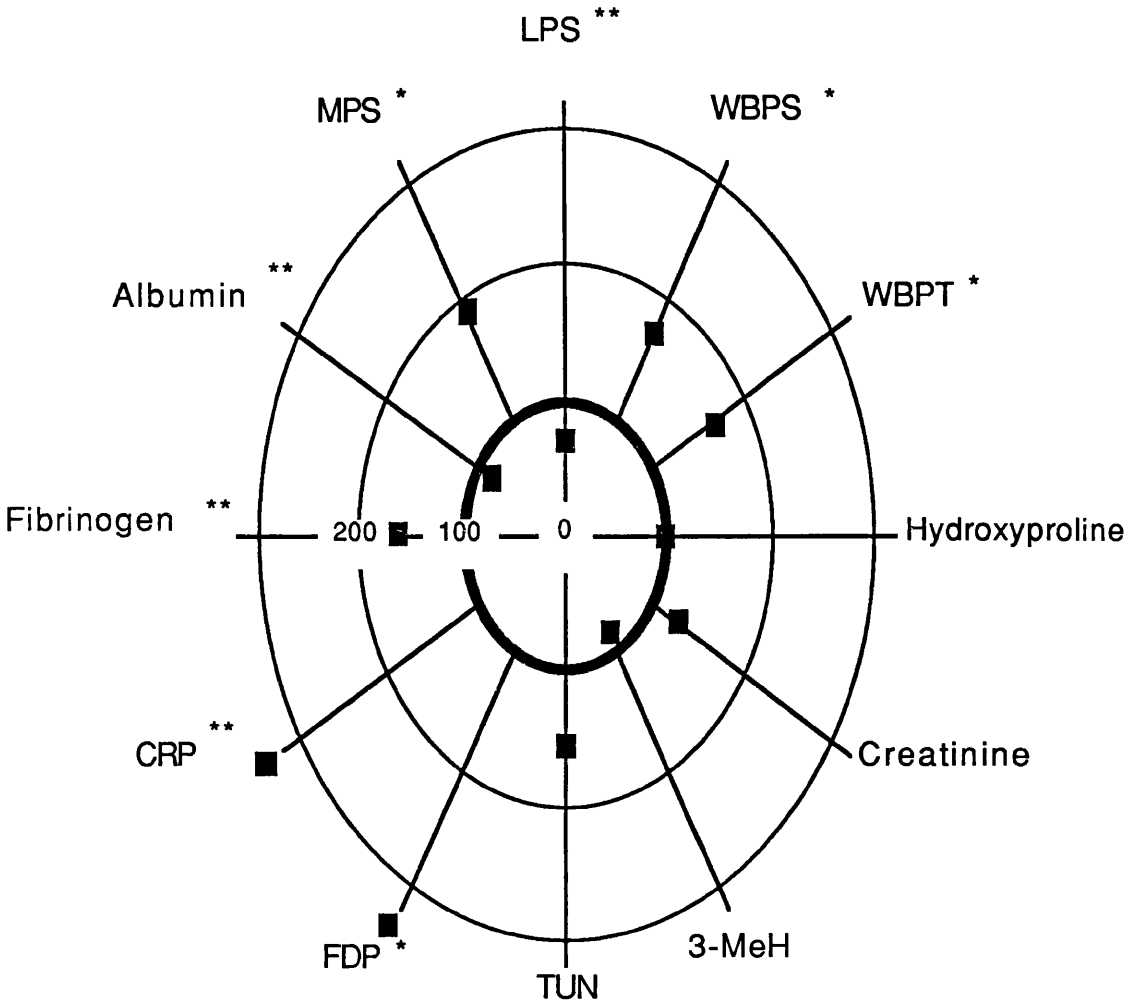


Figure 8.1 Protein synthesis and metabolites in weight-losing cancer patients. The mean value in the cancer group was normalised to the mean value in the control group (100%). * $p<0.05$, ** $p<0.01$.

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