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The Effect of Insulin and Amino Acids on Muscle and Whole Body Protein Turnover in Patients with Type I Diabetes

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M.D.

University of Edinburgh

1990

I dedicate this Thesis to Gerry, my wife.

Contents

Summary

Deficiency of insulin results in a net loss of lean body mass and wasting of muscle. The defect of protein turnover underlying the loss of skeletal muscle could be a reduction in the rate of protein synthesis, an increase in the rate of protein breakdown or a combination of both. In the present work the abnormalities of protein metabolism occurring during insulin deficiency have been investigated in patients with type 1 diabetes, and the acute effects of insulin replacement, during different conditions of nutrient supply, have been investigated both in patients with type 1 diabetes and in healthy subjects.

Incorporation of L-[1-^{13}C]leucine into biopsied skeletal muscle, arteriovenous leg exchange of L-[^{15}N]phenylalanine and of L-[1-^{13}C]leucine, and net release of 3-methylhistidine from leg tissues were used to assess muscle mixed protein synthesis, muscle mixed protein turnover and myofibrillar protein breakdown, respectively. Whole-body protein turnover was assessed by tracer dilution using L-[1-^{13}C]leucine and L-[^{15}N]phenylalanine.

Infusion of insulin reduced skeletal muscle mixed protein breakdown, both when plasma amino acids were allowed to fall and when their concentrations were elevated by exogenous amino acid infusion. In the diabetic patients muscle protein synthesis was not increased by insulin infusion alone; infusion of insulin combined with amino acids apparently increased synthesis as assessed by phenylalanine exchange but not when assessed by either leucine exchange or incorporation into muscle. In the healthy subjects there was a faster rate of muscle protein synthesis, assessed by phenylalanine exchange, during combined insulin with amino acid infusion in comparison with the diabetic patients. In healthy subjects infusion of mixed amino acids alone exerted an anabolic effect on muscle protein synthesis with

little, if any, alteration in the extent of either mixed or myofibrillar muscle protein breakdown. In comparisons with postabsorptive healthy subjects, abnormalities of muscle protein synthesis and breakdown and of amino acid oxidation in were not identified in type 1 diabetic patients during insulin withdrawal.

In the diabetic patients infusion of insulin alone reduced whole-body protein breakdown but also reduced protein synthesis to an equivalent extent; there was no net benefit on protein balance. However, infusion of insulin combined with amino acids reduced protein breakdown and improved net balance with no change in synthesis. In contrast, in the healthy subjects the same doses of insulin combined with amino acids stimulated protein synthesis *in addition* to reducing protein breakdown. The effects of amino acids alone in healthy subjects was both to stimulate whole-body protein synthesis and to reduce protein breakdown.

These investigations suggest that insulin exerts an anabolic action by reducing protein breakdown; insulin is only able to increase protein synthesis during conditions of increased amino acid availability. Muscle protein synthesis was resistant to stimulation by insulin in type 1 diabetic patients.

Declaration

The work described in this thesis was entirely undertaken by myself with the following specific exceptions:

1) The gas chromatography mass spectrometers were programmed by Dr C. M. Scrimgeour, Dr P. W. Watt and Mr K. Smith.

2) The isotope ratio mass spectrometer was programmed by Dr C. M. Scrimgeour, Mr K. Smith and Ms A. Wood.

3) Preparative gas chromatography of amino acids from muscle protein hydrolysates was undertaken by Mr K. Smith.

4) Amino acid concentrations in extracts from plasma and intramuscular water were determined by either Mr B. Weryk, University of Dundee, Mr C. Keenan, Peterborough District Hospital, Peterborough, U.K. or Dr P. Stehle, Institut Für Biologische Chemie Und Ernährungswissenschaft, Universität Hohenheim, Stuttgart, F.R.G.

5) Assistance was obtained from Ms S. Brumfitt and Ms A. Wood in preparation of samples for gas chromatography mass spectrometry.

6) Preparation and testing of sterile glucose solutions for infusion was kindly undertaken by Mr Cook, Pharmacy Department, Ninewells Hospital and Medical School.

Acknowledgements

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Mr C. Keenan, Peterborough District Hospital, Peterborough, U.K. and Dr P. Stehle, Institut Für Biologische Chemie Und Ernährungswissenschaft, Universität Hohenheim, Stuttgart, F.R.G. kindly undertook analysis of amino acid concentrations. Mr Cook, Pharmacy Department, Ninewells Hospital and Medical School kindly prepared the sterile potato-starch derived glucose solutions.

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Finally utmost thanks are due to the healthy volunteers and diabetic patients who volunteered to take part in these arduous studies, and to Dr R. W. Newton and Dr A. D. Struthers who allowed me to study their patients.

Abbreviations

APE	atoms percent excess
ATP	adenosine triphosphate
BCAA	branched-chain amino acid
BCAT	branched-chain amino transferase
BCKA	branched-chain keto acid
BCKAD	branched-chain keto acid dehydrogenase
DNA	deoxyribonucleic acid
eIF	eucaryotic initiation factor
GTP	guanosine triphosphate
IGF-I	insulin-like growth factor I
IMW	intramuscular water
Met-tRNA$_i^{met}$	initiator methionyl-tRNA$_i^{met}$
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid

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Chapter 1. INTRODUCTION

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1.1 Summary

The aim of the work described in this thesis was to define the effects of insulin on protein turnover both in muscle and in the whole-body. A specific aim was to identify under what conditions of substrate availability (i.e. amino acids and glucose) insulin was anabolic. An additional aim was to identify differences that existed in protein turnover in type 1 diabetic patients compared to healthy nondiabetic subjects. Chapter 1 covers concepts of protein turnover and amino acid metabolism. The focus on the literature is of the effects of insulin on protein metabolism, both *in vivo* and *in vitro*. Because a wide range of metabolic effects result from insulin deficiency and from hyperinsulinaemia the possible effects of these alterations of substrate and hormone concentrations on protein metabolism are considered in detail. In particular, the effect of alteration in amino acid concentrations on protein metabolism is considered in detail in this introduction and is a major focus in the present studies. A number of additional effects, eg immobilisation, which may play a role in the protein and amino acid economy of diseased subjects are considered in this chapter.

1.2 The Dynamic Nature of Protein

Protein is continuously renewed but different proteins are replaced at different rates. The term *protein turnover* should ideally be strictly confined to protein synthesis and protein breakdown although occasionally is used to include leucine oxidation. The whole-body protein turnover rate is a collective measure of the rates of turnover of all body proteins and it predominantly represents pools of proteins which turnover rapidly. The term *flux* is the rate of flow of an amino acid through the free amino acid

pool, not only in the pathways of protein synthesis and breakdown, but also in the pathways of intermediary metabolism of the amino acid, in uptake from diet and in *de novo* synthesis. In the models employed to calculate amino acid flux, oxidation and rates of protein synthesis and breakdown, excretion of small amounts of amino acid in faeces and urine and metabolism of amino acids in pathways such as formation of nucleotides and porphyrins are usually considered to be of little quantitative importance and thus are not taken into account.

1.2.1 Mechanism of Protein Synthesis

Information of the amino acid sequence required to produce proteins is contained in the genetic material as double-stranded DNA (Figure 1.1) (Waterlow *et al*, 1978a; Kimball and Jefferson, 1988). Proteins are coded for by sequences of base pairs, termed exons, joined by segments which do not code, introns. In the production of protein a mRNA copy of the DNA is produced by *transcription*. The mRNA transports the amino acid sequence codes from the nucleus to the site of protein synthesis. Amino acids are located correctly in sequence in relation to the mRNA by tRNA, to which they are acyl-bound and by polyribosomes as the process of *translation*. Translation of the information in mRNA into protein comprises the three steps of initiation (which appears to possess an important rate limiting function) (Figure 1.2) (Pain 1986), elongation and termination. After synthesis the protein may undergo further processing, e.g. partial proteolysis or glycation.

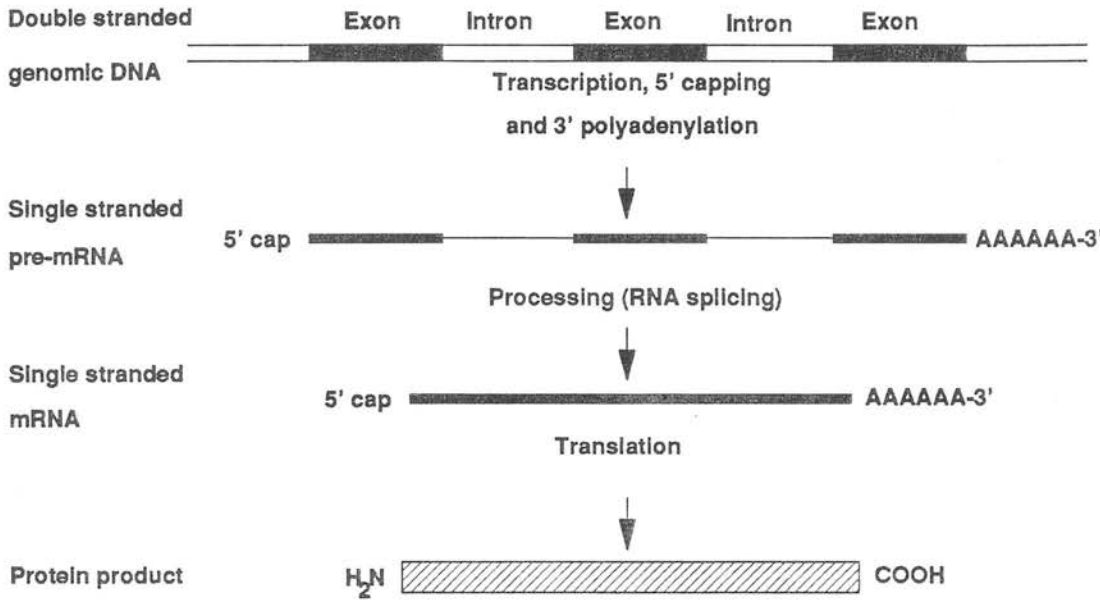

Figure 1.1. Transcription of the genetic material to pre-mRNA, processing to mRNA and translation to the protein product

1.2.2 Mechanisms of Protein Breakdown

There are a number of distinct mechanisms whereby proteins are hydrolysed to their constituent amino acids (Finley and Varshavsky, 1985; Mayer and Docherty, 1986; Kettelhut *et al*, 1988). The most extensively studied system is the *lysosome*. The membrane bound lysosome contains acidic proteases (cathepsins) and acid hydrolases. The lysosome predominantly degrades membrane proteins and glycoproteins but may be involved in the hydrolysis of cytoplasmic proteins during conditions of insulin deficiency and poor nutritional conditions. For muscles *in vitro* maintained under optimal conditions of nutrition, tension, amino acid availability and with insulin the

lysosome contributes little to proteolysis (Furuno and Goldberg, 1986; Lowell *et al*, 1986).

Calcium dependent proteases are responsible for marked proteolytic activity at neutral pH (Furuno and Goldberg, 1986). The extent that this system is important in proteolysis in healthy resting muscle is not clear but it appears to play an important role in damaged muscle (Kettelhut *et al*, 1988).

A system of energy dependent proteases has been identified which reduces in activity when oxidative phosphorylation and gluconeogenesis are inhibited. This *ATP dependent system* is shown to breakdown myofibrillar components. Part of the system may be a heat stable polypeptide, ubiquitin (Fagan *et al*, 1987). This becomes conjugated at its carboxyl terminal to the protein substrate at ε-amino groups on lysine residues of the protein, thus marking the protein for hydrolysis by a large enzyme complex, Ubiquitin-Conjugate-Degrading-Enzyme. This may be the most important system for protein breakdown in normal cells (Lowell *et al*, 1986). It is not clear at the present time if the ATP dependent system is largely dependent on ubiquitin tagging or if it predominantly functions separately.

1.3 Basic Concepts about Proteins and Amino Acids

Body protein in a normal man of 70 kg amounts to 10-11 kg, of which 50 to 65% is muscle (Cahill, 1970). Skeletal muscle represents approximately 40% of total body mass, or 70% of lean body mass, and is composed of approximately 18% protein. In skeletal muscle 65% of protein exists in the contractile elements, actin and myosin.

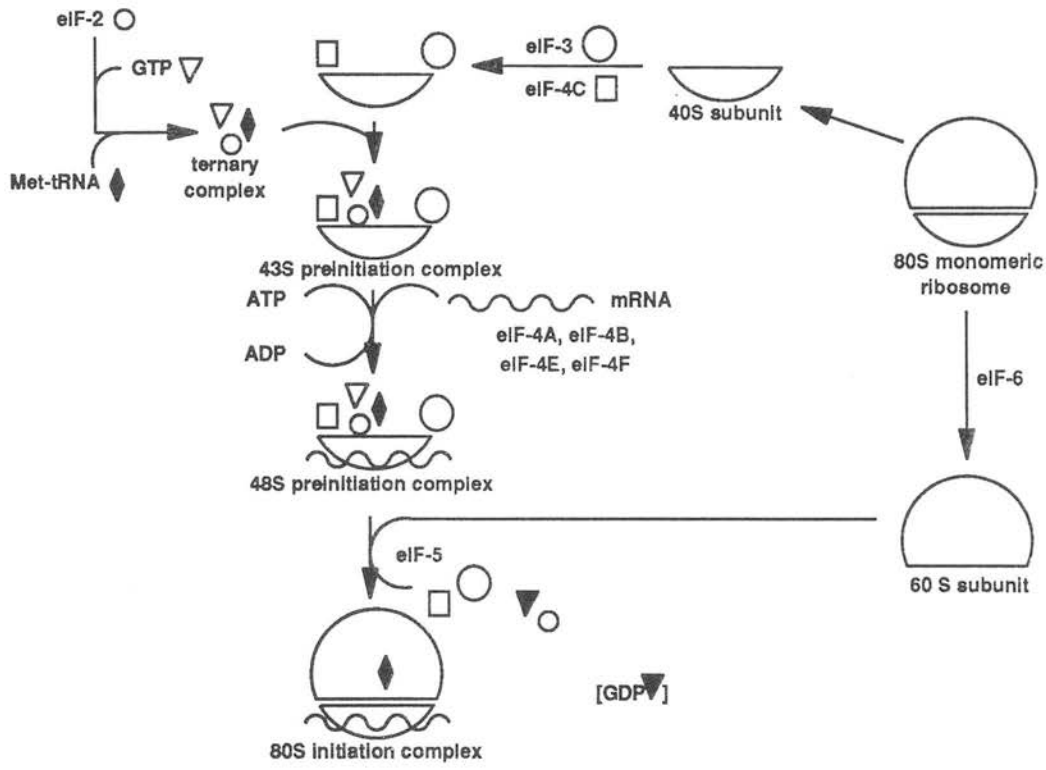

Figure 1.2. Schematic diagram of initiation of protein synthesis (Kimball and Jefferson, 1988). First a ternary complex is formed from eukaryotic initiation factor-2 (eIF-2), guanosine triphosphate (GTP) and initiator methionyl-tRNA$_i^{met}$ (Met-tRNA$_i^{met}$). The ternary complex binds to a 40S ribosomal subunit complexed to other initiation factors. With the hydrolysis of ATP and binding of additional initiation factors and mRNA, a 48S preinitiation complex is formed which enables the 60S ribosomal subunit to bind, forming an initiation complex. By altering the phosphorylation state of the initiation factors, and hence their ability to bind and form these complexes, insulin and other factors may modulate initiation.

Amino acids may be present *free* or *bound* as protein. Free amino acids are incorporated into proteins by protein synthesis and are released from it by protein breakdown (Figure 1.3). Amino acids may therefore enter the free amino acid pool by

release from protein, from the diet and, for the dispensable (or non-essential) amino acids by *de novo* synthesis.

Skeletal muscle has both mechanical and metabolic functions. Most of the total protein bound amino acids are present in muscle and about 50% of the free amino acids in addition (Munro, 1970). Skeletal muscle can therefore be considered a reservoir of amino acids which are available for a variety of metabolic processes. They may thus be used as *substrates* for the synthesis of protein, either locally or essentially in every other tissue in the body (Waterlow *et al*, 1978b). The amino acids may act as *precursors* for gluconeogenesis to enable glucose homeostasis during starvation (Cahill, 1970; Daniel *et al*, 1977). The gluconeogenic amino acids, alanine and glutamine, efficiently transport fuel from muscle to the liver and enter glucose synthesis by means of the glucose-alanine cycle (Figure 1.4) (Felig, 1975). Intermediary metabolites of branched-chain amino acids may act as substrates for fatty acid synthesis (Bender, 1985). Amino acids may act as substrate for further intermediary metabolism by certain tissues, for example glutamine uptake by gut as

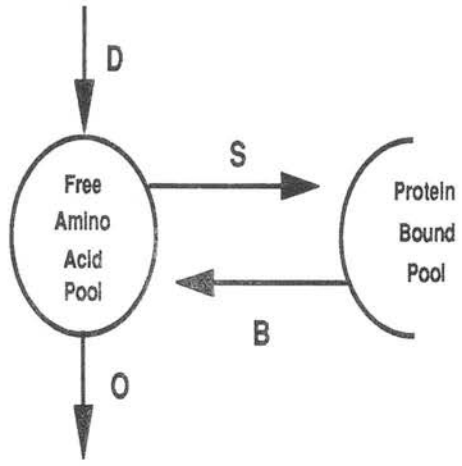

Figure 1.3. Two pool model of protein turnover; D, diet; S, Synthesis; B, Breakdown; O, Oxidation

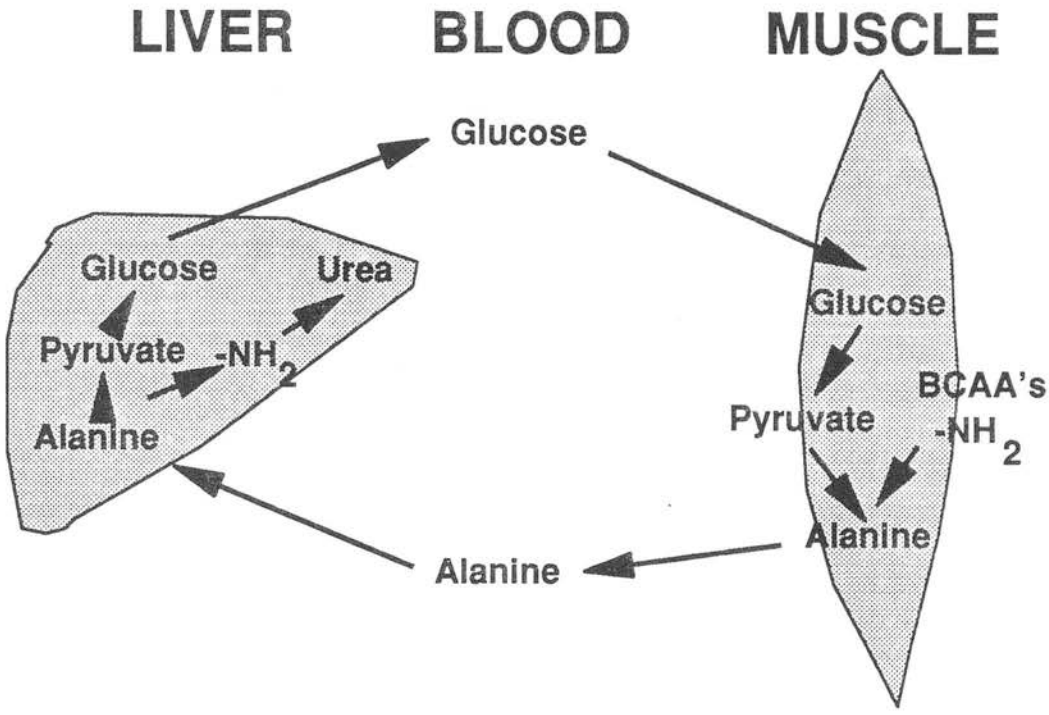

Figure 1.4. Glucose - alanine cycle

a source of nitrogen used in the synthesis of alanine (Matsutaka *et al*, 1973), in the kidney for ammonia synthesis (Cahill, 1970) and as a fuel in white cells, etc. AN futher function is as buffer during acid-base alterations, eg occurring in starvation when no cation accompanies ketone, bicarbonate, sulphate and phosphate excretion, glutamine is metabolised by the kidney to glutamate and the ammonium ion is excreted in the urine (Pitts *et al*, 1965; Golden *et al*, 1982).

1.4 Free Amino Acid Pools

The following facts concerning free amino acids have been pointed out by Munro (Munro, 1970): Free amino acids account for only 0.5 to 1% of the total amino acids in the body. Free amino nitrogen is predominantly in the form of only four of the eight dispensable amino acids, namely alanine, glutamate, glutamine and glycine. Half of the body free pool of amino acids is present in skeletal muscle which accordingly accounts for the largest proportion of the total body free amino acids of all tissues. For the dispensable amino acids in particular, the concentrations of the free amino acids within cells are much higher than their respective concentrations in plasma indicating the existence of either a concentration mechanism by transport processes which must ultimately be energy dependent (eg glutamine which uses the sodium gradient) or due to binding (eg taurine). The proportions of the individual amino acids within the free pool does not relate to their proportions within the protein bound amino acid pool. Specifically, the branched-chain amino acids compose 20% of protein but only 1% of the free amino acid pool.

1.4.1 Factors Influencing the Size of the Free Pool

Dietary intake is the major factor causing frequent and substantial alterations in the size of the free amino acid pool. Protein turnover is a factor which only has a net contribution to the free protein pool when synthesis and breakdown are not equal in magnitude. For the non-essential amino acids *de novo* synthesis occurs. Transamination of pyruvate to alanine and the amidation of glutamate to glutamine, are both important in the transport of amino-nitrogen from muscle to the liver (Felig

et al, 1969; Felig and Wahren, 1974). Changes in the concentrations of amino acids may, therefore, reflect changes in the extent of amino acid oxidation and of gluconeogenesis. The transport kinetics of the amino acid at the cell membrane may play a part in regulating the free amino acid pool. However, for most amino acids this transport process is rapid with a high rate of exchange between the plasma and the intracellular free pools. This results in an intracellular free pool with a short half-life. Amino acid oxidation regulates the size of the free pool.

1.4.2 Amino Acid Patterns

The plasma and free intramuscular concentrations and the ratio of intramuscular to plasma concentrations, in man, are shown in Table 1.1. Data for rat muscle are shown in Table 1.2.

There is no fixed relationship between the concentrations of amino acids in the bound and free amino acid pools (Munro, 1970), the ratios varying over 20 fold. Therefore, the patterns of amino acids in the free pool and their alterations may not *necessarily* reflect changes in the protein bound pool. During protein synthesis the relative amounts of the amino acids used may vary markedly from their relative distribution in the free pool. The differences in the patterns between the free and the bound pool are maintained by a number of mechanisms. Dietary protein generally has an amino acid composition similar to that of human proteins and, accordingly, to the mix of amino acids used in protein synthesis and released by protein breakdown. Changes in the extent of oxidation and the transmembrane transport of each amino acid may also play roles. The k_m for most *trasaminases* are above the range of normal

*Table 1.1. Plasma and Intramuscular Water (IMW) Free Amino Acid Concentrations in Man**

	Plasma (μmol/l)	IMW (μmol/l)	Ratio (IMW/Plasma)
Indispensable			
Histidine	80	370	4.6
Isoleucine	60	110	1.8
Leucine	120	150	1.2
Lysine	180	1150	6.4
Methionine	20	110	5.6
Phenylalanine	50	70	1.4
Threonine	150	1030	6.8
Valine	220	260	1.2
Dispensable			
Alanine	330	2340	7.3
Arginine	80	510	6.5
Asparagine	50	470	9.5
Citrulline	30	40	1.6
Cysteine	110	180	1.6
Glutamate	60	4380	74
Glutamine	570	19450	34
Glycine	210	1330	6.5
Ornithine	60	300	5.1
Proline	170	830	4.9
Serine	120	980	6.9
Taurine	70	15440	220
Tyrosine	50	100	2.0

*Bergström *et al*, 1974.

amino acid concentrations so that catabolism is to some extent driven by supply of substrate (Krebs, 1972). It follows, therefore, that if a substrate has a high steady state concentration (e.g. lysine and threonine) its oxidative system is likely to possess a low activity (Krebs, 1972). Conversely, the relatively low concentration of branched-chain amino acids in the free pool indicates that their oxidative enzymes have a high capacity and normal activity.

1.4.3 Influence of Diet on Free Amino Acid Pool

Protein deficient diets produce a reduction of up to 50% in the concentration of indispensable amino acids in plasma, with increases of the same order in the dispensable amino acids (Lunn, 1976). It may seem paradoxical, therefore, that starvation results in increases in the plasma indispensable amino acids with reductions in the dispensable amino acids (Munro, 1970). Starvation also increases the concentrations of indispensable amino acids in muscle, presumably because of reduced incorporation into proteins and increased protein breakdown (Millward *et al*, 1974), the branched-chain amino acids being particularly sensitive to such dietary modulation. In protein deficiency in man the branched-chain amino acids are specifically reduced by the greatest extent (Holt *et al*, 1963). Conversely, during fasting the branched-chain amino acids are increased to the greatest extent in muscle (Millward *et al*, 1976). Amino acids with a high ratio of bound/free are most sensitive to changes induced by dietary influences and alterations in amino acid turnover eg branched-chain amino acids which together constitute 20% of muscle protein.

*Table 1.2. Bound and Free Amino Acid Concentration in Rat Muscle**

	Protein (μmol/g net weight)	Free	Ratio (Protein/Free)
Indispensable			
Histidine	26	0.39	67
Isoleucine	50	0.16	306
Leucine	109	0.20	556
Lysine	58	1.86	31
Methionine	36	0.16	225
Phenylalanine	45	0.07	646
Threonine	60	1.94	31
Valine	83	0.31	272
Dispensable			
Alanine	111	2.77	40
Arginine	67	0.25	269
Aspartate (+amide)	110	1.13	97
Glutamate (+amide)	148	9.91	15
Glycine	117	1.94	60
Serine	74	1.96	38
Tyrosine	36	0.14	226

*Fern, 1975

1.4.4 Dynamic Nature of the Free Amino Acid Pool

The free amino acid pool of the rat can be calculated to turn over about every hour (Munro, 1970). In man the indispensable amino acid pool is smaller but because of the greatly reduced rate of protein turnover and thus a reduced flux the overall result is that it turns over more slowly than that of the rat (Bergström *et al*, 1974). Different amino acid pools turn over at different rates: that of leucine, which has a relatively high ratio of bound/free (Table 1.2), turns over about once every 60 min but in contrast lysine, with a lower ratio, takes ten hours. In some tissues free amino acid turnover is relatively rapid, eg in jejunum which has a turnover rate of 25 %/day, in contrast to muscle with a turnover rate ten-fold slower.

1.4.5 Transport Kinetics of Free Amino Acids

The rate of uptake of most free amino acids to human muscle is directly proportional to their plasma concentrations (Banos *et al*, 1973; Lundholm *et al*, 1987). It is conceivable that amino acid delivery might limit rates of protein synthesis in man with the amino acid having the smallest ratio of rates of delivery to incorporation into protein possibly constraining protein synthesis under conditions of high amino acid demand, such as during growth spurts or periods of tissue repair after injury, if dietary amino acid intake was concurrently reduced. For leucine, the blood concentration of which is about 0.12 mmol/l, if blood flow to total body muscle is approximately 1 l/min and protein synthetic rate is about 1.5 %/day (Halliday *et al*, 1988) then the daily total blood leucine delivery (170 mmol) is about 3.4 times the total amount of leucine incorporated into protein daily. Although this calculation suggests that it might be possible for the rate of muscle protein synthesis to exceed the rate of leucine delivery under certain conditions with greatly accelerated protein

accretion, this must be considered unlikely if account is made of leucine released from muscle by protein breakdown. However, if hyperinsulinaemia concurrently reduces amino acid release via protein breakdown thus reducing plasma (Fukagawa *et al*, 1985; Fukagawa *et al*, 1986; Tessari *et al*, 1986b) and muscle amino acid concentrations (Alvestrand *et al*, 1988), it is possible that amino acid delivery might restrict protein synthesis (Castellino *et al*, 1987). The foregoing analysis is fair but it should be emphasized that it only takes account of the broadest scheme of requirements. It may be that if transport, synthesis or breakdown occur in distinct compartments then the analysis may be to simplistic. Similarly it does not take into account the possible signalling roles of specific amino acids.

1.5 Assessment of Protein Turnover

Changes in muscle bulk and lean body mass may be measured by computed axial tomography (Häggmark *et al*, 1978; Bulcke *et al*, 1979; Termonte *et al*, 1980; Maughan *et al*, 1984; Buckley *et al*, 1987), magnetic resonance imaging (Andrew, 1980), whole-body potassium (^{40}K) counting (Preston *et al*, 1985), water (^{3}HHO or ^{2}HHO) dilution (Jensen *et al*, 1988a), total body electrical conductivity (Segal *et al*, 1985), densitometry (Durnin and Womersley, 1974) and anthropometry (Durnin and Womersley, 1974) but with these techniques changes in lean body mass and muscle size may only be determined over periods of weeks. Measurement of urinary excretion of creatinine or 3–methylhistidine may also be used as a measure of muscle mass. The principal techniques available for assessing protein turnover are:

a) Nitrogen balance techniques where the dietary nitrogen intake and urinary output of nitrogen are measured (and the faecal and sweat outputs of nitrogen either

measured or estimated) allowing an estimate of the *net protein balance* but neither protein synthesis nor breakdown (turnover in the true sense).

b) Urinary release of 3-methylhistidine, generally expressed as the ratio of 3–methylhistidine/creatinine, is an index of total myofibrillar protein breakdown in the whole-body (Ballard and Tomas, 1983).

c) 3-Methylhistidine release from leg tissues, measured as the product of arteriovenous concentration differences and blood flow, which may be a more specific assessment of skeletal muscle myofibrillar protein breakdown (Rennie and Millward, 1983).

d) Use of single dose of an isotope labelled amino acid to assess turnover or flux of the amino acid in the free amino acid pool from the rate of dilution of the tracer (Figure 1.5) (Henriques *et al*, 1955; Reilly and Green, 1975; Umpleby *et al*, 1986; Cobelli *et al*, 1987; Bier, 1989).

e) Estimation of protein turnover from dilution of [^{15}N] in metabolism end products (urea or ammonia) from labelled dispensable amino acids, usually [^{15}N]glycine (Sprinson and Rittenberg, 1949; Olesen *et al*, 1954; Fern *et al*, 1981; Waterlow, 1981).

f) Continuous infusion of a labelled amino acid to assess the turnover or flux of the amino acid in the free amino acid pool (Figure 1.6) (O'Keefe *et al*, 1974; Matthews *et al*, 1980; Clarke and Bier, 1982).

g) Incorporation of labelled free amino acid into the bound amino acid pool within tissue or plasma proteins (Figure 1.6), either *in vitro* or *in vivo*, (Schoenheimer *et al*, 1942; Halliday and McKeran, 1975; Rennie *et al*; 1982).

h) Use of a single dose of tracer combined with a large dose of non-labelled amino acid to flood the free amino acid pool and thereby label the intracellular amino acid

compartments with tracer to a similar extent as the accessible amino acid in plasma (Garlick *et al*, 1989a).

i) Simultaneous employment of arteriovenous phenylalanine tracer exchange and amino acid balance techniques to assess skeletal muscle protein breakdown and synthesis (Gelfand and Barrett, 1987; Thompson *et al*, 1989).

j) Measurement of tissue ribosome content to assess the capacity for protein synthesis (Millward *et al*, 1973) or measurement of polyribosomal profile in tissue biopsy samples to assess translational activity (Hammarqvist *et al*, 1988; Kimball and Jefferson, 1988; Wernerman *et al*, 1989).

Details of the techniques employed in the present investigations are covered in the next chapter.

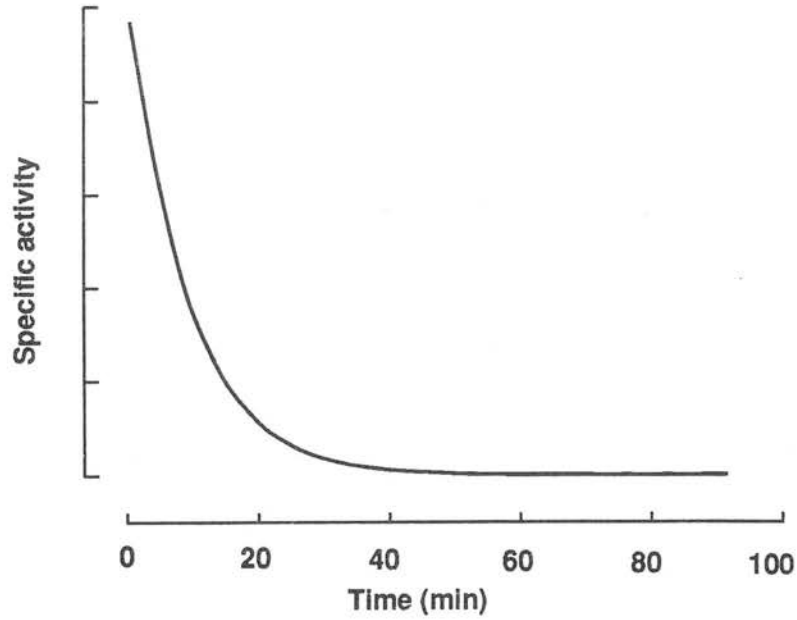

Figure 1.5. Schematic representation of the exponential decay in plasma specific activity following a single injection of a radioactive tracer

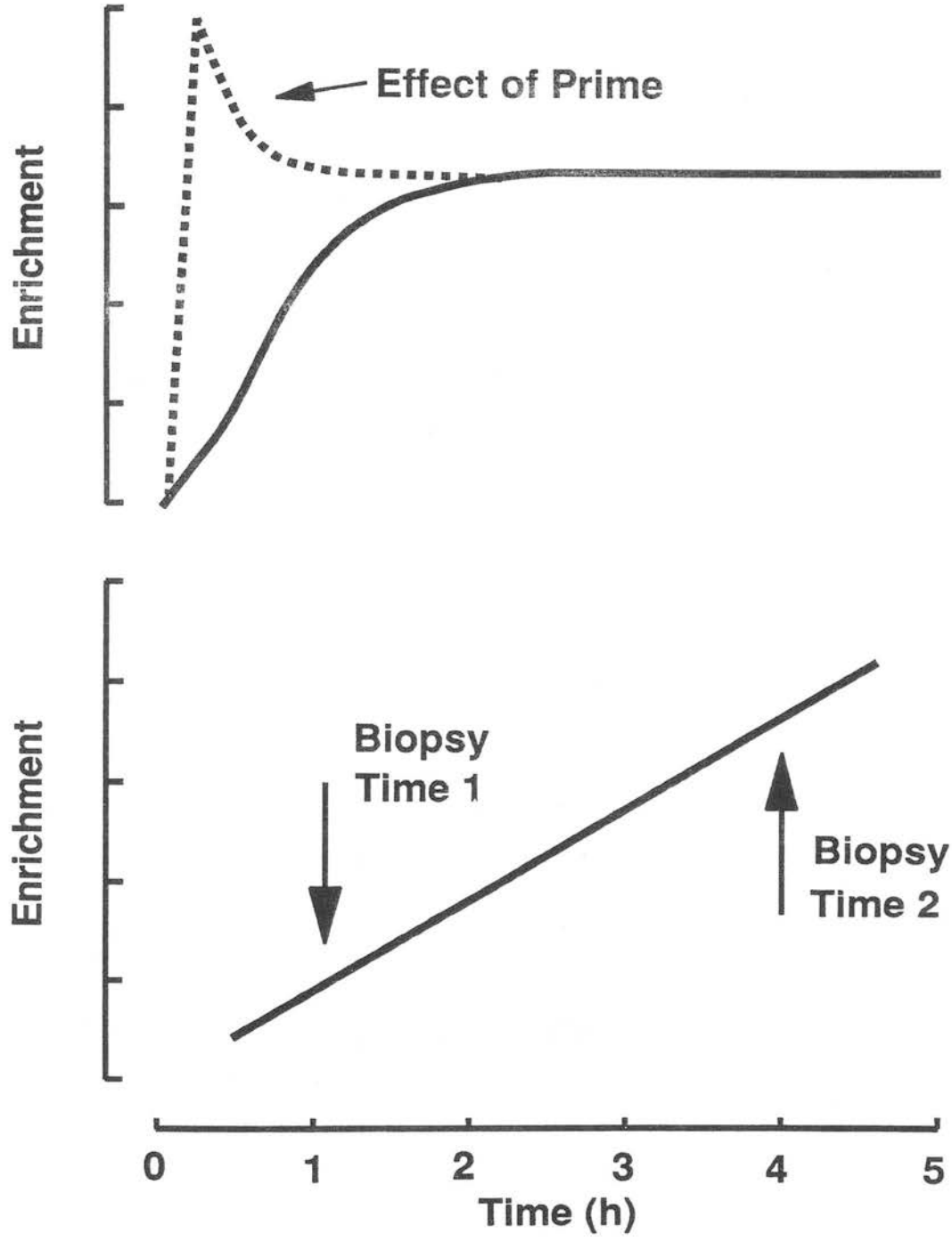

Figure 1.6. Upper panel: Schematic representation of a continuous infusion of stable isotope labelled tracer. Lower panel: Incorporation of the tracer into muscle

1.6 Maintenance, Growth and Wasting of Lean Body Mass

Lean body mass is maintained when protein synthesis and breakdown are in balance. Growth occurs when protein synthesis exceeds protein breakdown; protein breakdown may be reduced or increased in magnitude (Figure 1.7). Conversely, wasting occurs when breakdown exceeds protein synthesis; protein breakdown is not necessarily increased (Rennie, 1985).

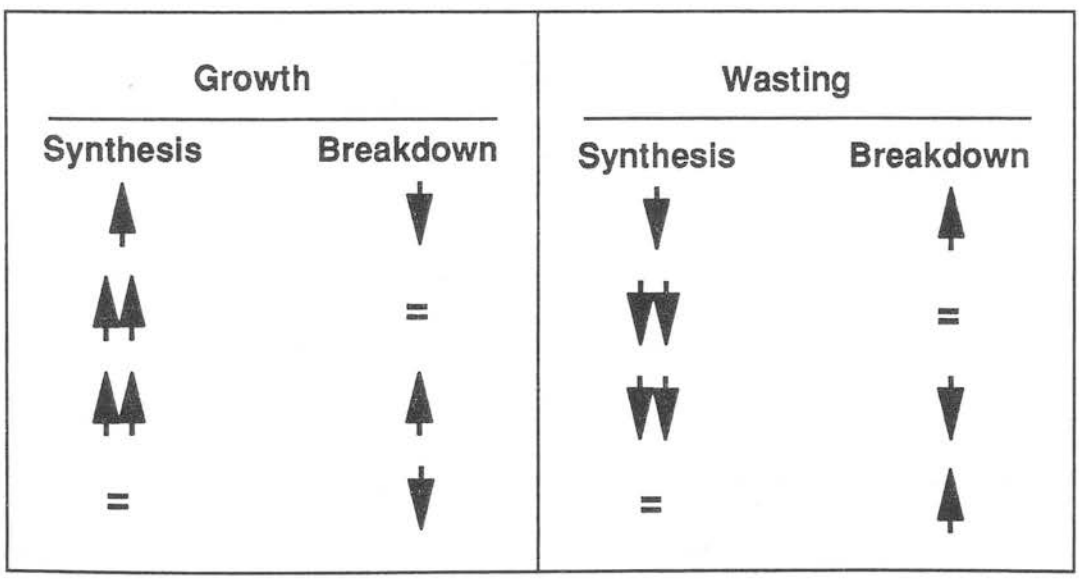

Figure 1.7. Hypothetical mechanisms of growth and tissue wasting

1.6.1 Mechanisms of Growth of Lean Tissues

Growth can be occur when rates of protein breakdown are increased; under these conditions protein synthesis must be markedly elevated if growth is to occur. This situation is encountered in the growth periods of immature animals where rates of protein synthesis (Garlick *et al*, 1989b) and breakdown are higher than in adults of the species on a fractional basis (Millward, 1980a; Millward, 1980b). The increased protein breakdown may allow remodelling of muscle rather than enabling growth to take place (Millward *et al*, 1975; Maruyama *et al*, 1978). During refeeding in malnourished rats both protein synthesis and protein breakdown increase (Young *et al*, 1971). Cardiac hypertrophy, which develops in such conditions as systemic hypertension and aortic stenosis, is associated with both increased protein synthesis and breakdown, resulting in a net protein synthesis (Gudbjarnason *et al*, 1964; Zak and Fischman, 1971; Morkin *et al*, 1972). It follows that a finding of increased protein breakdown does not necessarily indicate impairment of growth.

In contrast to the changes of mass of cardiac muscle, increases in liver tissue, which may occur after liver resection are associated with both increased protein synthesis and reduced protein breakdown (Scornik, 1974; Swick and Ip, 1974).

Reduced protein breakdown is demonstrated in a rapidly growing strain of rat (Bates and Millward, 1978). Alteration of thyroid status by administration of triiodothyronine at low levels to rats after thyroidectomy is shown to stimulate normal growth despite reduced protein synthesis; the balance between synthesis and breakdown was maintained by markedly reduced protein breakdown (Brown and Millward, 1983).

1.6.2 Mechanisms of Wasting of Lean Tissues

In contrast to growth, for wasting to occur protein breakdown must exceed protein synthesis. Analogous to the circumstances for growth, there are a number of possible mechanisms whereby wasting might occur (Figure 1.7).

Millward and colleagues have put forward the idea that alteration in protein synthesis by muscle tissues is the dominant pathophysiological mechanism which *facilitates* muscle wasting in a number of distinct conditions (Millward *et al*, 1976; Rennie *et al*, 1983). However, during increased protein synthesis there may be a paradoxical *adaptive* increase in protein breakdown which tends to oppose the effect of the changes in protein synthesis. Such changes occur during muscle hypertrophy induced by exercise (Laurent *et al*, 1978) and the opposite changes occur during starvation where both rates of protein synthesis and breakdown decrease (Millward and Waterlow, 1978). It is not yet established that synthesis is of principal importance in regulating tissue mass and certainly this does not appear to be the case for all other tissues. In liver protein breakdown appears to be of greater importance in regulating liver size than does protein breakdown (Garlick *et al*, 1973; Emery *et al*, 1986).

A full pattern has not been described for the abnormalities of protein synthesis and breakdown occurring in different pathophysiological situations in man where muscle wasting occurs. However, there is information about the pattern of muscle protein turnover found in a number of specific conditions. In patients with cardiac cachexia muscle protein breakdown is increased (Morrison *et al*, 1988c). In contrast in a number of other conditions where muscle wasting occurs protein breakdown is not

increased but muscle protein synthesis is reduced. These conditions include patients on their first day after operation, when undergoing abdominal surgery (Rennie *et al*, 1984); patients with Duchenne muscular dystrophy (Rennie *et al*, 1982a) and myotonic muscular dystrophy (Halliday *et al*, 1985); patients with untreated thyrotoxicosis (Morrison *et al*, 1988a) and patients with emphysema (Morrison *et al*, 1988b). In cancer patients with muscle wasting, not only is muscle protein synthesis reduced but in addition muscle protein breakdown is reduced (Lundholm *et al*, 1982).

1.6.3 Contribution by Muscle to Whole-body Protein Turnover

Information about the extent to which different tissues contribute to whole-body protein synthesis in man is limited. Muscle probably contributes from 25-50% of whole-body protein turnover in man (Munro, 1970; Rennie *et al*, 1982b; Nair *et al*, 1988a; Chapter 4); The revised data published by Rennie and colleagues (Halliday *et al*, 1988) suggests that the figure of 25%, calculated with amended methodology, may be a better estimate than the original value of 50%. It follows that changes in muscle protein metabolism may not be evident by alterations of whole-body protein turnover if opposing changes occurred in other tissues in the body. As protein turnover in many other tissues is more rapid than that in muscle, changes in protein turnover in liver and the splanchnic region might overshadow any changes occurring in muscle (Sakamoto *et al*, 1983; Emery *et al*, 1984).

1.6.4 Factors Which Induce Acute, Severe Muscle Wasting

In attempting to develop a complete picture of the control of muscle protein metabolism it must be noted that there are a number of factors which have acute and severe effects on muscle protein metabolism but which play little if any part in the physiological regulation of muscle mass in man. Such factors include absence of neural influences (Afting *et al*, 1981); severe infection or components of infection including lipopolysaccharide endotoxins (Wannemacher *et al*, 1971; Powanda, 1977; Fish and Spitzer, 1984; Filkins, 1985; Jepson *et al*, 1986) and damage occurring during raised intracellular calcium concentrations (Jackson *et al*, 1984).

1.7 Physiological and Pathophysiological Regulation of Protein Turnover by Feeding, Fasting and Substrate Supply

There is an extensive body of knowledge about the factors which modulate protein turnover in animals but for man such information is limited. There may be species differences with regard to the effects of any stimulus in animals or man and, in addition, many, if not most, experiments of the regulation of amino acid metabolism in animals have been undertaken in young growing animals which may behave differently from adult man. It, therefore, cannot be determined to what extent the information available from animal studies pertains to man. Many studies have employed *in vitro* experiments and even in these where human tissue has been used there may be major differences between the response of the isolated (often dying) tissue and of the intact animal.

I plan in this account to concentrate on information available from *in vivo* human studies but will also include information from animal studies, both *in vivo* and *in vitro*, whenever pertinent information from human studies is either unavailable or is limited by the methodology that can be employed in man. An additional rationale for considering the results from *in vitro* and *in vivo* animal studies is that where accord exists with the results from studies in man the information may be considered secure. In contrast where the results are discrepant there may be methodological or theoretical deficiencies which would render any results invalid.

1.7.1 The Effect of Feeding and Fasting

A net uptake of amino acids by muscle must occur during feeding (although it is difficult to document in man *in vivo*) and a net release occurs during fasting (Elia and Livesey, 1983). Studies of the incorporation of [1-^{13}C]leucine into quadriceps in healthy man show that protein synthesis is greater during feeding than during fasting (Rennie *et al*, 1982b; Halliday *et al*, 1988). These results have been confirmed by the *in vivo* application of a technique based on the exchange of [1-^{13}C,^{15}N]leucine by human forearm tissues (Cheng *et al*, 1985; Cheng *et al*, 1987). In addition these authors showed that feeding resulted in increased leucine deamination, reamination and oxidation and improved the net balance between synthesis and breakdown, but their results suggested that feeding had no effect on the rate of mixed muscle protein breakdown. The lack of any effect of feeding on muscle protein breakdown is also shown by Tracey and colleagues who assessed myofibrillar protein breakdown by limb release of 3-methylhistidine during parenteral feeding (Tracey *et al*, 1988). Feeding

of steak in man was shown to induce net amino acid uptake by the leg but this effect is partly offset by an increased net keto acid output (Elia and Livesey, 1983).

Both the muscle biopsy based and the arteriovenous exchange-based techniques have as a theoretical disadvantage the possibility that changes in the plasma concentration of the amino acid tracee, leucine, which occur during feeding might alter the tracer equilibrium between the accessible plasma pool and that at the site of incorporation of precursors for protein synthesis within cells. Increases in amino acid transport occurring at increased amino acid concentration (Lundholm *et al*, 1987) result in increased tracer and tracee exchange between plasma and the intracellular pool (Layman and Wolfe, 1987) and a closer approximation by the plasma enrichment to the intracellular enrichment. Estimates of intracellular amino acid metabolism during postabsorptive conditions, therefore, tend to be underestimated when the plasma amino acid enrichment or specific activity is used as a basis for calculations but underestimated to a lesser extent during feeding. It is follows that the tracer dilution technique employed in most studies tends to overestimate the increases in protein synthesis during feeding. Where the keto acid of leucine, a measure of the intracellular leucine labelling (Matthews *et al*, 1982; Schwenk *et al*, 1985a; Layman and Wolfe, 1987; Horber *et al*, 1989), was used to define precursor pool enrichment in the biopsy-based studies (Rennie *et al*, 1982b; Halliday *et al*, 1988) overestimation of the effects of feeding on protein synthesis would occur to a much lesser extent, if at all.

Garlick and collaborators, employing a flooding dose technique to minimise the differences in tracer enrichment between the plasma compartment and the precursor

pool for protein synthesis within the cell and, thereby, reduce the uncertainty about precursor labelling, have reported higher fractional protein synthesis rates in skeletal muscle in man (Garlick *et al*, 1989) than Halliday and colleagues (Halliday *et al*, 1988). Garlick found no effect of feeding on muscle protein synthesis in man using the flooding dose technique but *did* when the constant infusion technique was used (P.J. Garlick, personal communication). It remains to be determined if the addition of large quantities of leucine during these studies may have had some independent effect and masked any effects of feeding and fasting.

In fasted growing rats an effect of refeeding on muscle protein synthesis occurs after only 1 h (Garlick *et al*, 1983), results concordant with the results of the studies in man by Rennie and by Halliday. *In vitro* studies with muscle suggest that deficiency of nutrient supply both depresses protein synthesis and increases protein breakdown (Li and Goldberg, 1976; Li *et al*, 1979).

In contrast to the situation in muscle, loss of protein from liver during fasting does not occur as a result of a decrease in protein synthesis (Garlick *et al*, 1973, Garlick *et al*, 1975) in animals. Feeding causes a marked depression in the rate of liver protein breakdown (Conde and Scornik, 1976). The effect of feeding to increase protein synthesis in muscle and to reduce protein breakdown in liver (and possibly in muscle), might not be reflected in the results of in measures of whole-body protein breakdown. Liver protein turnover is much faster than muscle protein turnover and changes in liver protein turnover might overshadow any changes in muscle.

Mixed feeding is shown to increase whole-body protein synthesis in man (Garlick *et al*, 1980; Motil *et al*, 1981a; Motil *et al*, 1981b; Clugston and Garlick, 1982; Rennie *et al*, 1982b; Clague *et al*, 1983; Hoffer *et al*, 1985; McNurlan *et al*, 1987),

although not in all reports (Young *et al*, 1987; Beaufrere *et al*, 1989). Feeding, in most of these reports, reduced whole-body protein breakdown (Motil *et al*, 1981a; Motil *et al*, 1981b; Clugston and Garlick, 1982; Hoffer *et al*, 1985; Young *et al*, 1987; Tracey *et al*, 1988; Beaufrere *et al*, 1989) but no effect was reported by some authors (Garlick *et al*, 1980; Rennie *et al*, 1982b; Clague *et al*, 1983; Young *et al*, 1987). These measures of the effect of feeding are also subject to the uncertainties of precursor labelling detailed above although use of an α-ketoisocaproate-based model in a number of the studies may minimise this problem. In some studies substantial errors due to recycling of trace may have occurred: this problem, identified by Schwenk and colleagues (Schwenk *et al*, 1985b), leads to underestimation of protein synthesis where synthesis was measured sequentially following a period of fasting during prolonged tracer infusion. Failure to take account of metabolism of ingested leucine or protein by gut and by liver generally results in overestimates of the reduction in protein breakdown occurring during feeding. However, in studies where simultaneous intragastric and intravenous tracers were infused (Nissen and Haymond, 1986; Tessari *et al*, 1988; Beaufrere *et al*, 1989) reductions in whole-body protein breakdown are reported to occur with feeding. An additional source of error with most of the studies referenced above is failure to measure or account for the decreased proportion of bicarbonate which is fixed during feeding (Hoerr *et al*, 1989). Where this was accounted for feeding is reported to reduce whole-body protein breakdown but to have no effect on whole-body protein synthesis (Beaufrere *et al*, 1989).

Prolonged fasting, for four days, is shown to increase whole-body leucine flux or proteolysis (Jensen *et al*, 1988b), and to reduce skeletal muscle protein synthesis in man (Essén *et al*, 1989). The effect of fasting in the longer term is to reduce protein

utilization as a source of substrate for gluconeogenesis but such changes take about a week to be apparent (Kettelhut *et al*, 1988).

1.7.2 The Effect of Amino Acids

During infusion of mixed amino acids in man, increased net amino acid uptake occurred both in leg tissues and in the splanchnic bed (Gelfand *et al*, 1986). In that report muscle was estimated to remove about 25-30% of the total amino acid load infused and the splanchnic viscera (gut and liver) removed 65-70%. No account was taken of release of keto acids and amino acid metabolites in this study (Gelfand *et al*, 1986). In a subsequent study, where [1-^{14}C]leucine was infused to assess protein synthesis and breakdown during mixed amino acid infusion, amino acid infusion apparently increased skeletal muscle and visceral tissue protein synthesis and reduced skeletal muscle and visceral tissue protein breakdown (Gelfand *et al*, 1988); again the model employed in this study did not account for the intermediary metabolism of leucine.

Infusion of a solution of mixed amino acids is reported to increase whole-body protein synthesis (Castellino *et al*, 1987; Tessari *et al*, 1987; Pacy *et al*, 1988a). A uniform effect of the mixed amino acid infusion on whole-body protein breakdown was not reported: a decrease was found in two studies (Castellino *et al*, 1987; Pacy *et al*, 1988a) but no change was reported in one of the studies in which a different kinetic model was employed (Tessari *et al*, 1987) ie a double-primary pool model as opposed to a reciprocal-pool model.

Infusion of branched chain amino acids had no effect on either net leg protein balance or myofibrillar protein breakdown in either healthy subjects or in patients

with cardiac cachexia (Morrison *et al*, 1988c), and leucine had no effect on limb protein balance in man (Hagenfeldt *et al*, 1980; Eriksson *et al*; 1983). Abumrad and colleagues reported a transient and unsustained effect of leucine on forearm amino acid balance (Abumrad *et al*, 1982). Branched chain amino acids had no effect on protein synthesis assessed by ribosomal profile in man (Hammarqvist *et al*, 1988), but paradoxically leucine injection is reported to increase the proportion of muscle ribosomes as polyribosomes of fasted rats (Buse *et al*, 1979). Infusion of substantial amounts of leucine alone is reported to increase whole-body protein synthesis slightly in man (Schwenk and Haymond, 1987). In this study the investigators infused [4,5-^{3}H]leucine and α-keto[1-^{14}C]isocaproate rather than an amino acid tracer different from the substrate under test (Schwenk and Haymond, 1987). It is possible that alterations occured in the equilibrium between the tracer in plasma and in intracellular compartments due to increases in leucine concentration, and hence transmembrane transport; this must weaken any conclusions from the study.

In samples of human muscle studied *in vitro* a ten-fold increase in amino acid concentrations alone increased protein synthesis (Lundholm and Schersten, 1977). In addition complete mixtures of amino acids are reported to enhance protein synthesis in cell free systems (Tyobeka and Manchester, 1985). Leucine is reported to stimulate rat muscle protein synthesis *in vitro* (Buse and Reid, 1975) but this effect was not found in a study in rats *in vivo* (McNurlan *et al*, 1982). In a subsequent investigation both leucine and isoleucine were shown to have such an effect *in vitro*; in contrast neither other amino acids nor α-ketoisocaproate had such an effect (Hedden and Buse, 1982). It appears that the branched-chain amino acids (in particular leucine) are responsible for the effect of amino acid mixtures on stimulation of muscle protein

synthesis and of reductions in muscle protein breakdown (Buse and Reid, 1975; Fulks *et al*, 1975, Li and Jefferson, 1978; Adibi, 1980; Walser, 1984; May and Buse, 1989). The mechanisms whereby such effects are mediated are not known as the k_m for the charging of tRNA is likely to be much lower than the intracellular concentration of amino acids so that the TRNA is always fully charged (Shenoy and Roger, 1978; Flaim *et al*, 1982; Tyobeka and Manchester, 1985). The observation of a reduction in ribosomal subunits during provision of branched-chain amino acids (Li and Jefferson, 1978; Buse *et al*, 1979) indicates an effect of amino acids on initiation of protein synthesis.

In addition to an increase in muscle protein synthesis, mixtures of amino acids seem to reduce muscle protein breakdown in rodent muscles *in vitro* (Fulks *et al*, 1975; Frayn and Maycock, 1979). The effects of amino acids on liver protein turnover are both to reduce breakdown (Woodside and Mortimore, 1972; Mortimore *et al*, 1987) and to increase synthesis (Flaim *et al*, 1982).

There is a possible role of glutamine in controlling protein synthesis (MacLennan *et al*, 1987); this amino acid may play a key role in mediating the effects of corticosteroids (Muhlbacher *et al*, 1984) and of the injury process in general (Rennie *et al*, 1986) on protein turnover.

Garlick has suggested that the effect of amino acids is to increase the sensitivity of muscle protein to insulin (Garlick and Grant, 1988; McNurlan and Garlick, 1989). This may be an important mechanism whereby amino acids exert their anabolic effect but such a theory does not fully explain the effects of amino acids *in vitro* where insulin does not change.

1.7.3 The Effect of Substrates

There is little information available about the effects of different substrates on protein turnover in man.

β-*hydroxybutyrate:* The ketone body β-hydroxybutyrate has been suggested to increase muscle and whole-body protein synthesis and to reduce whole-body leucine oxidation in man (Nair *et al*, 1988b). It remains possible that this effect resulted from alterations in acid base homeostasis, a factor shown to have effects on protein turnover and amino acid oxidation in dogs (Rodriguez *et al*, 1989). There is no apparent effect of β-hydroxybutyrate on whole-body protein breakdown (Miles *et al*, 1983, Nair *et al*, 1988b).

Glucose: Infusion of glucose at 4 mg kg^{-1} min^{-1} is reported to reduce whole-body leucine oxidation and to decrease whole-body protein breakdown in healthy man with no effect on protein synthesis (Robert *et al*, 1984). In addition to an increase in plasma glucose concentration, plasma insulin increased (from 10 to 27 Mu/l) and there was a trend of reduced glucagon concentrations. It is possible that the effects of glucose provision were mediated by such alterations in hormones and were not a direct effect of glucose. In type 1 diabetic subjects during euglycaemic (5.9 mmol/l) and hyperglycaemic (15.9 mmol/l) insulin clamps, whole-body protein breakdown and leucine oxidation were greater during the latter study conditions (Robert *et al*, 1985). In these studies insulin was infused at 1.65 U/h during euglycemic studies and at 0.24 U/h during hyperglycemic studies which may have directly induced the differences in protein metabolism. Feeding excessive energy nutrients of either carbohydrate, fat or a mixture of both improved nitrogen balance and decreased leucine oxidation; these induced a reduction in protein breakdown but no alteration in protein synthesis (Motil

et al, 1981b). Experiments with rat muscle *in vitro* show that low concentrations of glucose in the perfusing media results in increased leucine oxidation than occurs during glucose replacement (Buse *et al*, 1972), and glucose alone has been reported to increase muscle protein synthesis (Hedden and Buse, 1982). In these studies lactate and pyruvate had similar effects to glucose, and protein synthesis was dependent on the cytoplasmic redox potential (i.e. ratio of NADH/NAD^+) (Hedden and Buse, 1982).

Triglycerides: Infusion of medium-chain triglycerides and long-chain triglycerides in man improves the net amino acid balance across forearm suggesting either increased protein synthesis or reduced proteolysis of skeletal muscle (Wicklmayr *et al*, 1987). Increases in plasma fatty acid concentrations in dogs reduced whole-body leucine flux and leucine oxidation and tended to reduce whole-body protein synthesis (Tessari *et al*, 1986c). Conversely, decreased plasma free fatty acid concentrations increased whole-body leucine flux and oxidation and decreased whole-body protein synthesis (Tessari *et al*, 1986c). The medium-chain fatty acid, octanoate, is reported to either increase (Buse *et al*, 1972; Paul and Adibi, 1986; Spydevold and Hokland, 1981; Wagenmakers and Veerkamp, 1982) or to decrease (Buffington *et al*, 1979; Buxton *et al*, 1984) leucine oxidation by rat muscle but to inhibit leucine oxidation in rat liver (May *et al*, 1980; Spydevold and Hokland, 1981). Octanoate is reported to reduce muscle protein synthesis *in vitro* (Wagenmakers and Veerkamp, 1984) and whole-body protein synthesis in dogs *in vivo* (Rodriguez *et al*, 1986).

1.8 The Effect of Hormones

Our understanding of the effects of hormones comes principally from studies in animals, often with *in vitro* preparations. While these enable information to be gained about apparently individual effects of hormones, the approach ignores the complicated interactions which exist between hormones, in both the regulation of their own secretion and that of other hormones and in interactions between them at end organs.

1.8.1 Insulin

Insulin acts on a membrane receptor which possesses intrinsic tyrosine kinase activity (Kahn and White, 1988). The events within the cell which occur after insulin binds are not clearly elucidated but appear to result from phosphorylation of intermediates.

In postabsorptive healthy man, regional infusion of insulin appears to reduce skeletal muscle protein breakdown, assessed by dilution of phenylalanine tracer, but has no obvious effect on skeletal muscle protein synthesis (Gelfand and Barrett, 1987). In addition no effect of systemic infusion of insulin was found on the rate of leucine incorporation into quadriceps muscle in type 1 diabetic subjects studied during insulin withdrawal and hyperglycaemia and during insulin replacement and euglycaemia (Pacy *et al*, 1989).

In contrast to these results from studies in man, insulin has been shown repeatedly to increase skeletal muscle protein synthesis in animals, both *in vitro* (Flaim *et al*, 1980; Jefferson, 1980; Airhart *et al*, 1982; Stirewalt *et al*, 1985; Kimball

and Jefferson, 1988) and *in vivo* (Hay and Waterlow, 1967; Pain and Garlick, 1974; Millward *et al*, 1976; Pain *et al*, 1983; Ashford and Pain, 1986a; Ashford and Pain, 1986b). The effect of insulin is to promote the initiation of translation (Fulks *et al*, 1975, Li and Jefferson, 1978; Kimball and Jefferson , 1988) and to maintain ribosomal levels predominantly by reducing degradation of ribosomes but also with an effect on synthesis particularly in heart muscle (Ashford and Pain, 1986a; Ashford and Pain, 1986b). Stimulation of gene expression for specific proteins (e.g. albumin) has been demonstrated *in vitro* (Lloyd *et al*, 1987; Dillmann, 1988). Despite reports of increased uptake of α-aminoisobutyrate (AIB), a non-metabolizable synthetic amino acid, into cells by insulin at supraphysiological concentrations (Kipnis and Noall, 1958) there appears to be no physiological effect of insulin *per se* on transport of all amino acid into cells (Hundal *et al*, 1989). In addition, there appears to be no effect of insulin on TRNA charging (Airhart *et al*, 1982; Stirewart *et al*, 1985).

The positive effect of insulin on protein synthesis has not always been confirmed, particularly in the fed state (Pain and Garlick, 1974; Oddy *et al*, 1987; McNurlan and Garlick, 1989). The reason for these differences in response to insulin in the different preparations are not apparent but may have occurred as a result of reduced viability of the *in vitro* preparations, or from differing substrate utilization of tissues studied *in vitro* or possibly from the alteration by streptozotocin of thyroid status in addition to induction of diabetes (Barrett and Gelfand, 1989). The net catabolic state of tissues studied in the *in vitro* situation may maintain a generous source of amino acids to supply protein synthesis, whereas the much reduced rates of protein breakdown which occur *in vivo* may limit amino acid availability and prevent

any increase in protein synthesis by insulin. In the rat protein synthesis by the liver is less sensitive to alterations of insulin than muscle (Hay and Waterlow, 1967; Pain and Garlick, 1974).

In type 1 diabetic subjects, withdrawal of insulin results in elevation of whole-body protein breakdown, an increase in plasma amino acid concentrations and an increase in rates of whole-body leucine oxidation (Nair *et al*, 1983; Robert *et al*, 1985; Tessari *et al*, 1986a; Umpleby *et al*, 1986; Nair *et al*, 1987a; Pacy *et al*, 1989). In contrast, in these subjects whole-body protein synthesis is neither reduced during insulin withdrawal, compared to healthy subjects, nor is it increased by insulin replacement. On the basis of these results it has been assumed that the effects of insulin in adult man are primarily on whole-body protein breakdown with no direct effect on whole body-protein synthesis.

Insulin is known to stabilize lysosomes and thereby limit muscle protein breakdown (Kettelhut *et al*, 1988). For muscle myofibrillar proteins lysosomes are not generally considered to be involved in protein breakdown (Lowell *et al*, 1986). Lysosomes are a site for proteolysis of non-contractile proteins of muscle and are important as a site of protein breakdown in liver. Insulin may reduce mixed skeletal muscle protein breakdown but have no effect on myofibrillar protein breakdown *in vitro* (Smith and Sugden, 1986), although other workers show a restraining effect of insulin on myofibrillar protein breakdown in diabetic rats (Smith *et al*, 1989). Whole-body protein synthesis may be reduced by about 30% by insulin infusion (Castellino *et al*, 1987; Tessari *et al*, 1987) suggesting that protein breakdown by lysosomes may account for about 30% of total protein breakdown in the body.

During studies in healthy man in which amino acids were infused with insulin to prevent insulin-induced hypoaminoacidaemia, insulin appeared to have no effect on whole-body protein synthesis; in contrast alteration of amino acid concentrations did alter whole-body protein synthesis (Castellino *et al*, 1987; Tessari *et al*, 1987; Flakoll *et al*, 1989). The concentration of amino acids within cells may not have been represented by the plasma concentrations and amino acid availability at a tissue level may not have been comparable during hyperaminoacidaemia in the absence and in the presence of insulin in these studies. In these studies insulin reduced whole-body protein breakdown (Castellino *et al*, 1987; Tessari *et al*, 1987; Flakoll *et al*, 1989).

1.8.2 Growth Hormone

Growth hormone causes children to grow (Tanner *et al*, 1977) and improves nitrogen balance in man (Manson and Wilmore, 1986; Clemmons *et al*, 1987; Ponting *et al*, 1988, Ziegler *et al*, 1988). In pharmacological doses in healthy man, growth hormone decreases whole-body leucine oxidation and increases whole-body protein synthesis but appears to have no effect on whole-body protein breakdown (Horber and Haymond, 1989). In growth hormone deficient children whole-body protein synthesis is depressed and growth hormone treatment rapidly increases whole-body protein synthesis (Richter *et al*, 1987). There is no published information about the effect of growth hormone on muscle protein turnover in man but in animals it appears to have a direct anabolic action by increasing muscle protein synthesis (Cameron *et al*; 1988). Most of the effects of growth hormone are mediated by insulin-growth factor I (Isgaard *et al*, 1989), but in addition insulin secretion is increased by growth hormone and this

may play an independent part in the mediation of growth hormone action on protein metabolism.

1.8.3 Insulin-like Growth Factor I

The effects of growth hormone are predominantly mediated by the two insulin-like growth factors I and II (IGF-I and IGF-II), factors postulated to exist on the basis of pioneering experiments by Salmon and Daughaday (Salmon and Daughaday, 1957). About half of IGF-I is produced by the liver (Daughaday and Rotwein, 1989). Almost all tissues have the capacity to express the growth factors (Han *et al*, 1988) and, therefore, they may function predominantly as paracrine rather than as endocrine agents. IGF-I acts on receptors with close structural homology to the insulin receptor (Rechler and Nissley, 1989). Only the relatively recent development of specific antibodies against IGF-I and its receptor have enabled a clear separation of IGF-I effects from these of insulin. The growth factor, IGF-I, possesses potent stimulatory activity on protein synthesis *in vitro* (Froesch *et al*, 1985). In contrast, when infused intravenously in rodents IGF-I apparently *decreased* whole-body protein synthesis and breakdown and *reduced* the rates of protein synthesis in a number of specific tissues (Jacob *et al*, 1989). These results may have resulted from use of supraphysiological doses, where the IGF-I was not associated with its specific binding protein, and a direct effect mediated via the insulin receptor may have resulted. An additional uncertainty with this piece of work is that the magnitude of the protein turnover rates are a few fold lower than would be expected in the rat. It has been shown that growth hormone has greater growth promoting effects than IGF-I suggesting that additional

factors other than IGF-I are important in mediating the effects of growth hormone (Skottner *et al*, 1987). Results of the effect of IGF-I and IGF-II on protein turnover in man are not available at present but the recent introduction of synthetic IGF-I by recombinant gene technology should stimulate research in this area.

1.8.4 Cortisol

The glucocorticoids, and cortisol in particular, have a negative effect on protein balance. In man short courses of cortisol increase whole-body protein breakdown (Beaufrere *et al*, 1989) and stimulate transfer of nitrogen from leucine to alanine and to glutamine with a resultant increased synthesis of these gluconeogenic amino acids (Simmons *et al*, 1984; Daurmaun *et al*, 1988). In subjects with longer-duration hyperglucocorticoidaemia from untreated Cushing's syndrome increased whole-body protein breakdown was not observed (Tessari *et al*, 1989). This overtly anomalous result may have arisen either as a result of expression of the leucine kinetic results after normalization for total body weight as opposed to lean body mass (due to decreased muscle and protein mass in patients compared to controls), or may relate to use of a leucine kinetic model where flux is derived from the sum of leucine and α-ketoisocaproate primary pool estimations. It is also likely that these different effects of cortisol in the longer term are the result of adaptive mechanisms such as increased insulin concentration and to a predominant action of glucocorticoids to impair protein synthesis. Wolfe has suggested that amino acid homeostasis may escape control by corticosteroids in physiological doses (Wolfe *et al*, 1989), but this view contradicts many published observations.

From the results of animal-based studies corticosteroids may both increase protein myofibrillar protein breakdown and decrease muscle protein synthesis (Rannels and Jefferson, 1980; Odedra *et al*, 1983; Kayali *et al*, 1987), but no effect on myofibrillar protein breakdown was shown by Beaufrere and colleagues in man (Beaufrere *et al*, 1989). Glucocorticoids decrease glutamine concentrations in plasma and muscle (Muhlbacher *et al*, 1984): this may be a possible mechanism whereby they action on protein turnover as glutamine appears to regulate protein turnover during injury (Rennie *et al*, 1986). Glucocorticoids are also known to reduce RNA concentration and to inhibit translation (Rannels and Jefferson, 1980) in animals and to decrease muscle ribosome concentration in man (Wernerman *et al*, 1989).

1.8.5 Adrenaline and Noradrenaline

Infusion of adrenaline in healthy man decreases muscle ribosome concentration (Wernerman *et al*, 1989) and decreases whole-body protein breakdown, leucine oxidation and stimulates *de novo* alanine synthesis (Miles *et al*, 1984; Kraenzlin *et al*, 1989). This may be a direct effect or possibly an indirect effect mediated by alterations in insulin concentration. Muscle protein breakdown appears to be reduced by adrenaline (Kraenzlin *et al*, 1989). There is less direct information about the effect of noradrenaline (Gelfand *et al*, 1984) and its possible effects have not been elucidated. An interesting possibility is that alteration of catecholamine concentrations by insulin infusion (Rowe *et al*, 1981; Liang *et al*, 1982) may mediate some of the metabolic effects of insulin. In rat muscle, β-agonists decrease protein breakdown (Li and Jefferson, 1977).

1.8.6 Thyroxine and Triiodothyronine

In man thyroxine excess results in a depression of skeletal muscle protein synthesis but unaltered myofibrillar protein breakdown, as assessed by the increased arteriovenous net efflux of tyrosine and unaltered 3-methylhistidine balance across the leg (Morrison *et al*, 1988a). Morrison and colleagues did not demonstrate any significant effect of hypothyroidism on leg protein balance but in animal studies hypothyroidism results in reduced protein synthesis by muscle (Brown *et al*, 1981), corrected by replacement treatment with triiodothyronine (Brown and Millward, 1983). Excess replacement results in increased muscle protein synthesis in rats (Brown and Millward, 1983) and in increased muscle protein breakdown (Brown and Millward, 1983; Barrett and Gelfand, 1989). The mechanism of this effect is not clear (Millward and Rivers, 1989) but may involve control of ribosome levels. In their study, Morrison and colleagues found impaired whole-body protein synthesis both in the thyrotoxic patients and in the hypothyroid patients. The differences between animal and man may relate to different durations of thyroid abnormality but a clear explanation is not available.

1.8.7 Glucagon

In man glucagon increases whole-body protein breakdown and leucine oxidation when insulin is deficient (Nair *et al*, 1987b) but not with insulin replacement (Fukagawa *et al*, 1990). Similar effects are reported in studies in pigs (Ostaszewski and Nissen, 1988). Although it has been suggested that glucagon inhibits muscle protein synthesis *in vivo* such an effect is only seen at pharmacological doses (Preedy

and Garlick, 1988) and probably has no physiological significance: there is no effect of glucagon on muscle ribosome concentration or on the proportion of muscle ribosomes as polyribosomes in man (Wernerman *et al*, 1989). In rats *in vivo* a deleterious effect of glucagon on muscle and whole-body nitrogen balance is seen, even in the presence of insulin (Almdal and Vilstrup, 1988).

1.8.8 Testosterone

Testosterone, administered in pharmacological doses, increases skeletal muscle protein synthesis in man, with no apparent effect on whole-body protein synthesis (Griggs *et al*, 1989).

1.8.9 Tumour Necrosis Factor-α and Interleukin-1

This polypeptide hormone, produced by lymphocytes or macrophages, appears to increase skeletal muscle protein breakdown (assessed by net amino acid efflux from the limb) in cancer patients but conversely to increase whole-body protein synthesis (possibly in liver) (Warren *et al*, 1987; Starnes *et al*, 1988). Studies undertaken with *in vitro* conditions have generally, but not universally, failed to confirm such an effect of increased muscle protein breakdown (Evans *et al*, 1989), suggesting that the effects of tumour necrosis factor-α (TNF) may be mediated indirectly, e.g. by increases in cortisol or glucagon. There is generally thought to be little or no effect of interleukin-1 (IL-1) on protein turnover but a related factor, interleukin-6 may have a stimulatory effect on muscle protein breakdown (Evans *et al*, 1989). However, IL-1 has been

suggested to increase muscle protein breakdown in some situations and the effect may be mediated by prostaglandin E_2 (Baracos *et al*, 1983) but this IL-1 was *not* pure.

1.8.10 Prostaglandins

Prostaglandin E_2 and $F_{2\alpha}$ increase protein breakdown in rat muscle *in vitro* (Rodemann and Goldberg, 1982). The inhibitor of prostaglandin synthesis, ibuprofen, inhibits the increased whole-body proteolysis seen in man during infusion of *E. Coli* endotoxin (lipopolysaccharide), but the effects may be indirect by alteration of the concentrations of the major stress hormones (U. Keller, personal communication).

1.9 The Influence of Exercise and Immobility on Protein Turnover

Strength training in young men increases skeletal muscle protein synthesis (K. Yarasheski and D.M. Bier, personal communication) and both improves net amino acid balance and reduces myofibrillar protein breakdown of the human limb (Albert *et al*, 1989). Functional electrical stimulation increases muscle protein synthesis in paraplegic patients (Pacy *et al*, 1988b). Immobilisation in a plaster cast decreased human muscle protein synthesis by a quarter and the authors estimated that muscle protein breakdown decreased in addition (Gibson *et al*, 1987; Gibson *et al*, 1988). The reduced muscle protein synthesis during immobilisation was abolished by functional electrical stimulation so that muscle wasting was abolished (Gibson *et al*, 1988; Gibson *et al*, 1989).

In the whole-body, exercise increases protein breakdown and leucine oxidation (Rennie *et al*, 1981; Albert *et al*, 1989).

1.10 Pathophysiological Regulation of Amino Acid Metabolism

There are clear details of the mechanisms of muscle and protein wasting in very few conditions in man. There is depression of skeletal muscle protein synthesis in muscular dystrophy (Rennie *et al*, 1982a; Halliday *et al*, 1985; Halliday *et al*, 1988): in these studies patients with end-stage muscle disease were studied (when muscle may be extensively replaced by connective tissue and when immobility may play a role in depressing muscle protein turnover) and these results may not be applicable to patients with the early stages of these diseases. In patients with cardiac cachexia myofibrillar protein breakdown is increased but whole-body protein turnover is depressed (Morrison *et al*, 1988c). In patients with hepatic cirrhosis there does not appear to be any detectable abnormality in the whole-body protein turnover rate (Millikan *et al*, 1985; Mullen *et al*, 1986) or in children with cystic fibrosis (Thompson and Tomas, 1987). Increased whole-body protein breakdown occurs in homozygous sickle cell disease (Badaloo *et al*, 1989).

1.9 The Effect of Trauma and Sepsis on Protein Metabolism

Surgical trauma decreases the breakdown rate of muscle myofibrillar proteins (Rennie *et al*, 1984) and reduces net amino acid release from the leg (Stjernström *et al*, 1986). In addition reductions in muscle protein synthesis occur (Wolfe *et al*, 1989). Leucine oxidation increases following injury, as does energy expenditure, but severe injury results in greater leucine oxidation than other energy-equivalent conditions suggesting a degree of breakdown of protein homeostatic mechanisms. This appears to be the result of liberation of amino acid stores in muscle perhaps with the beneficial

effect of making substrate available for synthesis of reactive proteins. The increase in leucine oxidation may be an inevitable consequence of the increased leucine concentration. The report of an effect of branched chain amino acids to reduce nitrogen loss (Cerra *et al*, 1983) appears unlikely as they would most likely undergo catabolism.

The counterregulatory hormones may be important in modulating the increased proteolysis of injury but the paracrine hormones and cytokines may be equally, if not more important (Wolfe *et al*, 1989). In sepsis, studied using a model with lipopolysaccharide from *E. Coli*, there is an increase in muscle protein breakdown and a reduction in muscle protein synthesis (Jepson *et al*, 1986) and when lipopolysaccharide is infused in man whole-body protein breakdown increases (U. Keller, personal communication).

1.11 The Present Studies

The aim of the studies detailed in this work was clarification of the role of insulin deficiency and its replacement in the modulation of protein synthesis. There has been intense interest by a number of workers in this area of research during the last few years and many of the references in the bibliography were not available when I started my work. Our initial hypothesis was that insulin exerted an anabolic effect on muscle by increasing muscle protein synthesis in man. In view of the profound reductions in amino acid concentrations which occur during hyperinsulinaemic conditions we expected no increase of either muscle or whole body protein synthesis when insulin was given alone but we anticipated an effect when insulin was administered with adequate quantities of amino acids and energy substrates.

Accordingly, we designed protocols to study the action of insulin and the effect of amino acid availability on muscle protein synthesis. We realized that there is a substantial body of evidence suggesting that the insulin-like growth factors may be more important than insulin and in addition non-hormonal influences, eg exercise may predominate in regulating protein turnover. A further aim was to further develop and refine a number of techniques for estimating muscle protein turnover. Specifically these included: a technique with anterior tibial muscle biopsy for measuring skeletal muscle fractional protein synthesis; assessment of leg protein turnover with using labelled-phenylalanine; and use of [1-^{13}C]leucine for assessing leg protein turnover and leucine metabolism.

Chapter 2. MODELLING PROTEIN TURNOVER AND AMINO ACID METABOLISM

CONTENTS

FIGURES

2.1 Stable Isotopes

The measurement of protein turnover *in vivo* was shown to be possible in a series of pioneering experiments with ^{15}N and ^{2}H labelled amino acids initiated in the 1930's (Schoenheimer and Rittenberg, 1938; Schoenheimer *et al*, 1939a; Schoenheimer *et al*, 1939b; Schoenheimer *et al*, 1942). In the last decade, there has been an increased impetus to investigation of amino acid metabolism *in vivo* in man due to the ready availability of a large range of commercially-produced stable isotope-labelled amino acids and the development of reliable and relatively inexpensive mass spectrometers.

The stable isotopes most commonly used to investigate human protein metabolism are ^{2}H (i.e. Deuterium or D), ^{13}C, ^{15}N and ^{18}O. There are substantial advantages to be gained in employing stable rather than radioactive isotopes for the investigation of human metabolism. There are no useful radioactive equivalents of either ^{15}N or ^{18}O. Stable isotopes do not decay and release no ionizing radiation, a particularly important point for studies in children, women in the reproductive years and young healthy people in general. Precautions are not required to protect the investigators handling these compounds from radioactive hazard. When analyzing stable isotope samples to determine the tracee molar enrichment only one measurement is required, whereas, the analysis of samples to determine radioactive tracee specific radioactivity requires separate measurements of radioactive and chemical concentrations, with the result that the coefficient of variation of the latter analysis is likely to be greater. A minor disadvantage is that these isotopes are ubiquitous and basal or background values must be measured and taken into account.

2.1.1 Tracer : Tracee Differential Metabolism

It is generally assumed that stable isotope labelled amino acids are metabolised in an identical way to their tracee. Proof supporting this assumption is deficient although indirect evidence is available. Where similar tracers are concurrently infused in the same subjects either minimal or no differences are found in metabolism of the tracers. For example we have found systematic differences of only 4% in the whole-body leucine flux rate in dogs when measured simultaneously with [1-^{14}C]leucine and [4,5-^{3}H]leucine (W. M. Bennet and M. W. Haymond, unpublished). It is not clear if such differences are due to *in vivo* differential tracer metabolism or alternatively to analytical imprecision. We have observed earlier retention times for [$^{2}H_{10}$]leucine compared with [^{12}C] or [1-^{13}C]leucine and also for [$^{2}H_{5}$-*phenyl*]phenylalanine compared with [^{14}N] or [^{15}N]phenylalanine during gas chromatography mass spectrometry of *tert*-butyldimethylsilyl derivatives on a capillary column (C. M. Scrimgeour and W. M. Bennet, unpublished). Similar effects are seen during high-pressure liquid chromatographic separation of labelled α-ketoisocaproate: α-keto[4,5-^{3}H]isocaproate elutes slightly before and α-keto[1-^{14}C]caproate slightly after unlabelled α-ketoisocaproate (B. A. Krom and F. F. Horber, personal communication). These differential isotope effects *in vitro* suggest that similar effects may occur *in vitro*.

Bacteria die when grown in $^{2}H_{2}O$ enriched higher than 50%, and systematic tracer:tracee differences must be accounted for when energy expenditure is measured with the doubly labelled water technique (i.e. when $^{2}HH^{18}O$ is administered) (Nagy, 1980). For example, vapour lost from the body contains 5% less deuterium and 1% less

^{18}O than body water. Foods may have different natural abundance of ^{13}C; this is most marked for sugars from different sources. Plants with C_3 photosynthesis (e.g. potato or sugar beet derived glucose) have less ^{13}C than plants with C_4 photosynthesis (e.g. corn derived glucose) (Schoeller *et al*, 1980). These observations collectively suggest that isotope fractionation may occur for labelled amino acids, but such an effect is likely to be of the order of a few percent at maximum.

2.2.1 Overview of Techniques Employed in the Whole-body

In the studies described here two stable isotope labelled amino acids (i.e. L–[1–^{13}C]leucine and L–[^{15}N]phenylalanine) were used with conceptually different modelling approaches to assess protein turnover in the whole-body and in skeletal muscle. These techniques provide different and complimentary measures of protein turnover in the whole-body, in anterior tibialis muscle, and in leg tissues (predominantly skeletal muscle). Since the metabolically active pools accessible to these distinct trace techniques were different and the model-dependent assumptions underlying them were variable, the results obtained may not always be in agreement or even comparable between techniques. Three important features of both amino acids are a) they are indispensable, b) the free pool of these amino acids is small compared to their respective bound pools (Table 1.2) and c) transport into cells, by the L–system transporter, is not dependent on endocrine regulation (Yudelevitch and Boyd, 1987; Hundal *et al*, 1989).

The following techniques were used to measure amino acid metabolism in the whole-body:

a) The stable isotope labelled amino acid L–[1–^{13}C]leucine was used with a plasma α–ketoisocaproate based open four-pool model to determine rates of whole-body leucine kinetics (Rennie *et al*, 1982b; Schwenk *et al*, 1985a; Bier 1989).

b) The stable isotope labelled amino acid L–[^{15}N]phenylalanine was used with a two-pool model to determine the rates of whole-body phenylalanine kinetics (Clarke and Bier, 1982).

2.2.2 Overview of Techniques Employed to Assess Skeletal Muscle Metabolism

Use of these stable isotope labelled amino acids also enabled measurements to be made of skeletal muscle protein turnover in leg tissues. These tracer techniques and, also, a non-tracer technique used to measure skeletal muscle protein turnover were as follows:

c) Anterior tibialis protein synthetic rate was determined from the incorporation of L–[1–^{13}C]leucine into protein obtained from muscle biopsy samples, during a continuous infusion of the tracer (Rennie *et al*, 1982b; Halliday *et al*, 1988).

d) Arteriovenous exchange of phenylalanine by the leg was determined using L–[^{15}N]phenylalanine. This technique provided measures of protein balance, protein synthesis and protein breakdown of skeletal muscle (Gelfand and Barrett, 1987; Thompson *et al*, 1989).

e) Arteriovenous exchange and oxidation of leucine by the leg was determined using L–[1–^{13}C]leucine. This provided measures of synthesis and breakdown of skeletal muscle (Cheng *et al*, 1985; Cheng *et al*, 1987).

f) Arteriovenous release of 3–methylhistidine from leg tissues was determined. This provided a semi-quantitative index of myofibrillar protein breakdown of skeletal muscle (Lundholm *et al*, 1982; Rennie and Millward, 1983; Rennie *et al*, 1983).

2.2.3 Application of Techniques

In all the studies undertaken whole-body leucine turnover was measured (technique No. a). Incorporation of leucine into skeletal muscle (No. c) was measured in on group of healthy subjects to test the effect of infusion of mixed amino acids and in one group of type 1 diabetic subjects to assess the effect of insulin during increased amino acid availability. In the remainder of the subjects whole-body phenylalanine kinetics were measured using L–[^{15}N]phenylalanine (No. b) and leg arteriovenous exchange of phenylalanine and leucine were measured (Nos. d & e respectively): these groups comprised two groups of healthy subjects where the effects of amino acid availability and the effect of insulin were investigated and one group of type 1 diabetic subjects where the effect of insulin and the effect of amino acids was investigated. Leg release of 3–methylhistidine (No. f) was also determined in one subgroup of healthy subjects to ascertain the effect of alteration of amino acid concentrations.

2.3.1 Whole-body Protein Turnover Determined with [1-^{13}C]Leucine: Equations for Steady State Conditions

When tracer-labelled amino acid is infused intravenously it is metabolised by the body in the same way as endogenous amino acids. Where the essential amino acid leucine (which is not synthesized *de novo*) is infused as the tracer, it mixes throughout the plasma space and disappears into cells. Within cells it may enter into protein synthesis, and become incorporated into protein, or it may undergo catabolism. Leucine is rapidly and reversibly deaminated (Figure 2.1) to its keto acid,

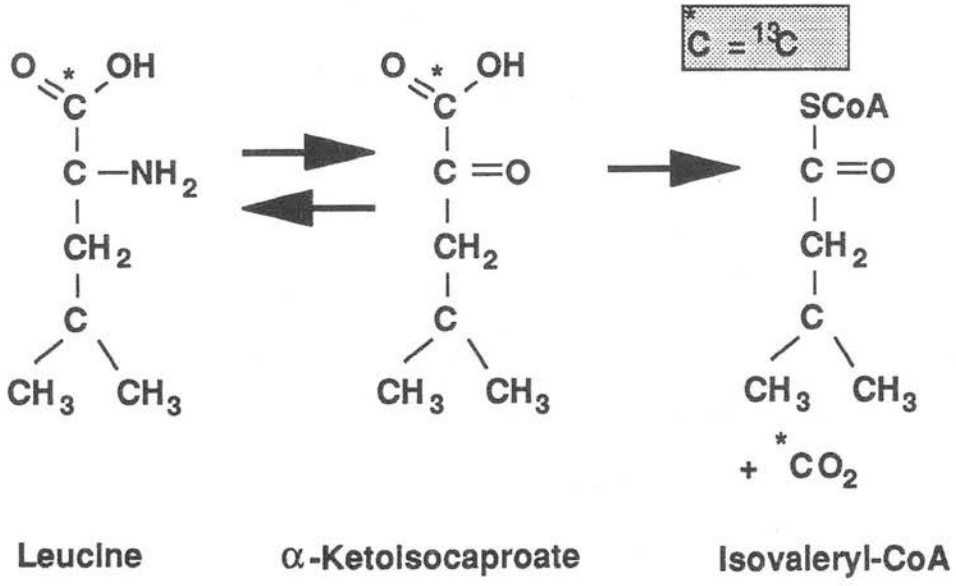

Figure 2.1. Leucine intermediary metabolism

α–ketoisocaproate (2–keto–4–methyl–pentanoate, 2–ketoleucine), by branched-chain amino transferase (BCAT) which is widely distributed in human tissues (Goto *et al*, 1977). Accordingly, the removal of nitrogen label from leucine in man is rapid, both in the whole-body (Matthews *et al*, 1981) and by muscle (Cheng *et al*, 1985; Cheng *et al*, 1987). Where a carbon or hydrogen label is used to label leucine, the label is not lost during transamination (Matthews *et al*, 1982) but the label may be diluted by unlabelled leucine and α–ketoisocaproate (Figure 2.1). The keto acid, α–ketoisocaproate, may undergo irreversible oxidative decarboxylation by branched-chain keto acid dehydrogenase (BCKAD) present on the inner mitochondrial membrane (May and Buse, 1989) and also widely distributed in man with about 60% in muscle (Khatra *et al*, 1977) (Figure 2.1). The ^{13}C label is lost during decarboxylation and $^{13}CO_2$ is produced. In the rat plasma concentrations of α–ketoisocaproate are about 15-35 μmol/l and the relative tissue to plasma concentration ratios are about 0.4 for muscle, 0.2 in heart and 0.3 in liver (Hutson and Harper, 1981).

The free tracer, both in plasma and in intracellular fluid, is diluted by non-labelled amino acids either released by protein breakdown or from the diet. The rates of these metabolic processes can be determined by measurement of the ^{13}C enrichment of plasma α–ketoisocaproate and of its oxidation product, carbon dioxide, excreted in breath and by comparison with the rate of infusion of tracer. The immediate metabolite of leucine, α–ketoisocaproate, is measured in preference to leucine on the assumption that this provides a closer measure of the tracer enrichment of leucine in the intracellular milieu (Matthews *et al*, 1982; Rennie *et al*, 1982b; Schwenk *et al*, 1985a; Layman and Wolfe, 1987; Horber *et al*, 1989). Based on an open four pool

α–ketoisocaproate-based model or reciprocal pool model (Figure 2.2) (Schwenk *et al*, 1985a; Bier 1989) the components of whole-body amino acid turnover can be calculated (Rennie *et al*, 1982b; Matthews *et al*, 1982); a leucine tracer is infused into the plasma leucine pool and plasma α–ketoisocaproate is sampled or alternatively a tracer of α–ketoisocaproate is infused and plasma leucine sampled, hence the term "reciprocal".

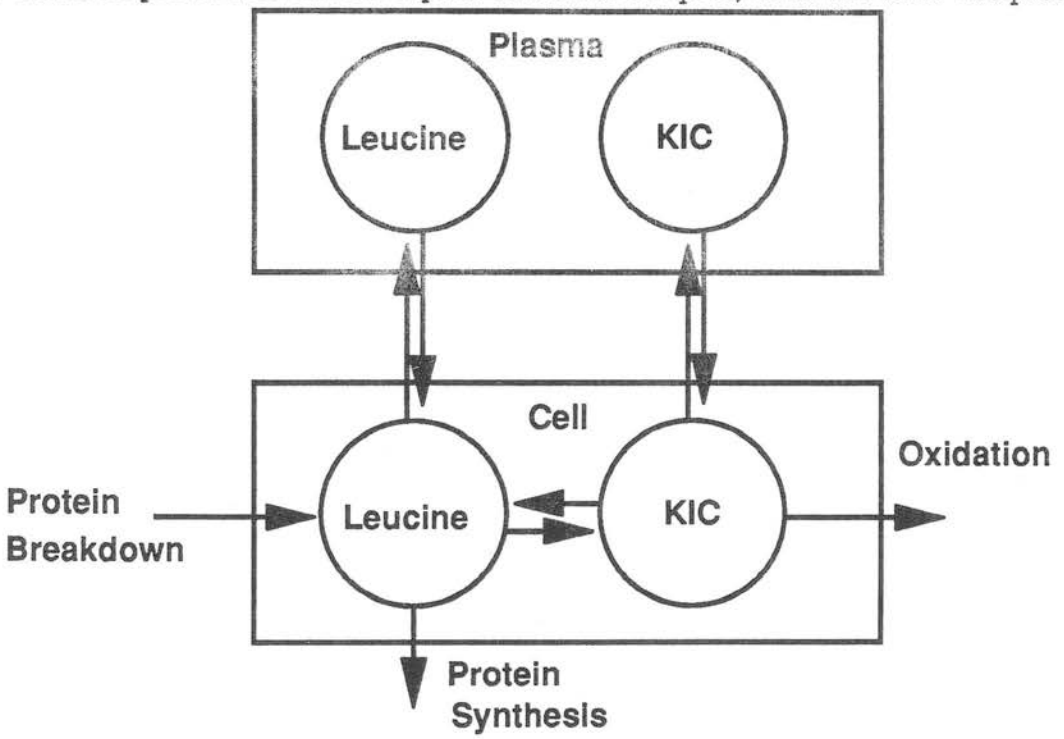

Figure 2.2. Open four pool or reciprocal pool leucine model (see text)

Leucine kinetics are calculated from the ^{13}C enrichment of α–ketoisocaproate in plasma and of CO_2 in breath after plateau conditions of labelling have developed during a primed continuous infusion of L–[1–^{13}C]leucine (Figure 1.6) (Matthews *et al*, 1980; Rennie *et al*, 1982b): the prime doses of both [^{13}C]leucine and sodium [^{13}C]bicarbonate are given to achieve plateau conditions more rapidly than by a

continuous infusion alone (Allsop *et al*, 1978; Matthews *et al*, 1980). The individual components of leucine turnover may be calculated from the following equation (O'Keefe *et al*, 1974) (Figure 1.3):

$$Q = S + O = B + D$$

Where:

Q = the rate of leucine turnover or *flux*,

S = non-oxidative leucine disposal or protein *synthesis*,

O = *oxidation* of leucine,

B = endogenous leucine appearance or protein *breakdown*, and

D = *dietary* intake of leucine.

The units of whole-body leucine kinetics are micromoles of leucine per kilogram total-body weight per hour ($\mu mol \bullet kg^{-1} \bullet h^{-1}$). This equation assumes that leucine disposal by routes other than protein synthesis and leucine oxidation is negligible. The equation is only valid for steady-state conditions where a true plateau exists for the ^{13}C enrichment of α–ketoisocaproate and the plasma concentrations of plasma leucine and α–ketoisocaproate are constant.

The flux or turnover of leucine is calculated from the following equation (Matthews *et al*, 1980; Rennie *et al*, 1982b):

$$Q = i \left(\frac{Ei}{Ep} - 1 \right)$$

Where:

i = infusion rate of L–[1–^{13}C]leucine in $\mu mol \bullet kg^{-1} \bullet h^{-1}$,

Ei = ^{13}C enrichment of the infused L–[1–^{13}C]leucine in atoms % excess (APE), and

E_P = ^{13}C enrichment of α–ketoisocaproate in plasma (APE) after tracer equilibration. Well mixed samples reflecting metabolism in all tissues in the whole-body are required and, therefore, arterialized venous samples are taken with the "hot-box" technique (Section 3.5).

The constant 1 is subtracted to correct for the rate of tracer infusion where a stable tracer is employed (Matthews *et al*, 1980; Tessari *et al*, 1985). This correction assumes that the absolute flux increases in proportion to the rate of tracer infusion and that the endogenous flux is unaltered. Where the rate of *i* is less than 10% of Q there appears to be no measurable effect of infusing the tracer either on the flux or oxidation of leucine (Tessari *et al*, 1985).

The rate of release of carbon dioxide (μmol of $^{13}CO_2 \bullet kg^{-1} \bullet h^{-1}$) from oxidation of the [1–^{13}C]leucine tracer is (Matthews *et al*, 1980):

$$F^{13}CO_2 = \frac{VCO_2 \bullet ECO_2}{W} \bullet \frac{60 \bullet 4.16 \bullet 10^4}{100 \bullet 0.81}$$

Where:

VCO_2 = Expiration rate of carbon dioxide (l/min at Standard Temperature and Pressure, STP),

ECO_2 = ^{13}C enrichment in expired carbon dioxide (APE) after isotope equilibration, and

W = body weight of the subject (kg).

The factors:

60 converts min to h,

$4.16 \bullet 10^4$ converts l of carbon dioxide (at STP) to μmol of carbon dioxide,

100 converts enrichment in atoms % excess to a fraction, and

0.81 takes account of the reduced quantity of carbon dioxide released in expired breath following tracer oxidation after passage through the bicarbonate pool, on account of fixation of carbon dioxide in tissues (Issekutz *et al*, 1968; James *et al*, 1976, Hoerr *et al*, 1989).

The rate of whole-body leucine oxidation ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) is:

$$O = F^{13}CO_2 \left(\frac{1}{Ep} - \frac{1}{Ei} \right) \cdot 100$$

From leucine flux (Q) and leucine oxidation (O) the whole-body non-oxidised leucine disposal (S) or leucine incorporation into protein (i.e. protein synthesis) is:

$$S = Q - O$$

Leucine release by protein breakdown (B) is calculated from flux (Q) and dietary intake (D):

$$B = Q - D$$

Where the studies are conducted in the fasted or postabsorptive state dietary intake is zero (D = 0) and:

$$B = Q$$

A number of assumptions are integral requirements to the use of the these equations based on the labelling of α–ketoisocaproate; these are:

a) There is no difference in the metabolism of ^{13}C-labelled leucine and α–ketoisocaproate compared with unlabelled endogenous leucine and α–ketoisocaproate.

b) There is negligible release of tracer-labelled leucine from protein after it has been incorporated by protein synthesis, i.e. there is negligible *recycling* of tracer. Schwenk and colleagues (Schwenk *et al*, 1985b) have reported that tracer recycling of up to 30% occurs in studies where tracer is infused for 24 h. However, recycling probably occurs to a minor extent, perhaps less than about 5% in studies of less than 10 h. It has been shown that the leucine flux rate in healthy postabsorptive subjects is not different at the end of 4 h in comparison to 8 h of study during continuous [1–^{13}C]leucine infusion (Nair *et al*, 1988b), suggesting that recycling is minimal during studies of this duration.

c) There are four pools of free leucine (Figure 2.2): a) free as leucine in the intravascular space and extracellular matrix, b) free as leucine within cells where rapid exchange occurs with intracellular α–ketoisocaproate (Pool c), which in turn rapidly exchanges with α–ketoisocaproate in the intravascular compartment (Pool d).

d) Transamination and reamination of leucine to α–ketoisocaproate is a very rapid process, such that intracellular free leucine and α–ketoisocaproate have the same tracer enrichment. Matthews and colleagues (Matthews *et al*, 1981) have demonstrated that in man the rates of transamination and reamination of leucine are fast in comparison with the oxidation of α–ketoisocaproate. Cheng and colleagues (Cheng *et al*, 1985; Cheng *et al*, 1987) have shown that this is also the case for human forearm.

e) The degree of dilution of the tracer or the *enrichment* of α–ketoisocaproate in plasma is representative of that of both α–ketoisocaproate and leucine at an intracellular level, in the precursor pools for α–ketoisocaproate oxidation and for leucine incorporation into protein, respectively, in all tissues throughout the body. At the present time information that this assumption actually holds true is not available for man. Animal studies, both *in vitro* and *in vivo*, of leucine incorporation into muscle suggest that the charging of leucyl-tRNA (i.e. the leucine precursor pool for protein synthesis) is predominantly from plasma or extracellular fluid (Davey and Manchester, 1969; Martin *et al*, 1977; Clark and Zak, 1981; Everett *et al*, 1981; Martin, 1981; Airhart *et al*, 1982). However, Schneible and colleagues (Schneible *et al*, 1981) reported conflicting observations from a study of chicken muscle *in vitro*: i.e. that leucine entering into protein synthesis was predominantly derived from leucine released by protein breakdown within the cell. They also reported that leucine entering oxidation derived predominantly from the extracellular milieu.

f) In subjects, in whom leucine was infused parenterally, it entered into cells, and mixed in the intracellular free leucine and α–ketoisocaproate pools before undergoing metabolism.

g) Estimates of CO_2 fixation obtained from studies of infusion of sodium [^{13}C]bicarbonate or sodium [^{14}C]bicarbonate are appropriate to estimate fixation of CO_2 released from leucine oxidation within mitochondria. In addition, these estimates are appropriate under all conditions of study. It practice it appears that a greater proportion of CO_2 is fixed from leucine oxidation (within mitochondria) than from bicarbonate infused into the plasma space, as assessed by greater isotope labelling of

glucose by incorporation of the CO_2 during gluconeogenesis (F. F. Horber, P. C. Butler and M. W. Haymond, personal communication); leucine oxidation, therefore, may be systematically underestimated by the general adoption of a 19% bicarbonate fixation value. In addition, bicarbonate fixation (on a proportional basis) is reduced during feeding (Hoerr *et al*, 1989) and may be altered by acid-base disturbances such as diabetic decompensation and by insulin replacement.

2.3.2 Whole-body Protein Turnover Determined with [1-^{13}C]Leucine: Kinetic Equations for Non-steady State Conditions

The equations described above are only suitable for calculation of whole-body leucine kinetics in the steady-state, i.e. when the plasma concentrations and ^{13}C enrichments of leucine and α-ketoisocaproate are constant. Under most conditions of study these conditions are met by giving appropriate priming doses of leucine and by allowing a period of about one hour or more after commencement of the tracer infusion before sampling. This period is adequate to allow a plateau to develop even after major perturbations have occurred in protein metabolism such as with intravenous infusion of mixed amino acid solutions. In postabsorptive healthy subjects plasma leucine concentrations tend to slowly increase because of breakdown from protein but the increase is negligible in comparison to whole-body leucine flux rates. It has been shown that the whole-body leucine flux rate is the same when measured after 4 h in comparison to 8 h of continuous infusion of [1-^{13}C]leucine in healthy postabsorptive subjects (Nair *et al*, 1987b).

In contrast to the steady state, in healthy subjects where insulin secretion is suppressed by somatostatin infusion, progressive increases occur for at least three hours in the plasma leucine concentration and in the whole-body leucine flux, such that plateau conditions are not attained (Nair *et al*, 1987b). When insulin is replaced, the reductions which occur in plasma leucine concentration and in whole-body leucine flux both in healthy subjects (Fukagawa *et al*, 1985; Fukagawa *et al*, 1986; Tessari *et al*, 1986b; Castellino *et al*, 1987) and in diabetic subjects (Tessari *et al*, 1986a; Nair *et al*, 1987) are such that the criteria for applying steady-state models may not be fulfilled within 3 to 4 hour study periods.

Alternative equations may be used to calculate whole-body leucine kinetics during non-steady state situations. These equations take account of the changes which occur in plasma leucine and α–ketoisocaproate concentration and the changes in ^{13}C enrichment of plasma leucine and α–ketoisocaproate. The equations were developed by Steele (Steele, 1959) for use in studies of glucose metabolism with radio-active tracers. The equations were adapted for use in stable-isotope studies by Miles and colleagues (Miles *et al*, 1983). The equations detailed here have been further modified by incorporation of a whole-body leucine volume of distribution of 0.6 l/kg (i.e. total body water). This is based on the assumption that leucine is distributed at an equivalent concentration throughout total-body water and that changes which occur in the concentration of plasma leucine reflect these occurring in leucine within all cells of the body. The original equations, which used the volume of distribution for extracellular water (0.15 l/kg), may be more appropriate for glucose metabolism studies. In contrast, the value of 0.6 l/kg should render the equations more

appropriate for calculating whole-body leucine metabolism as, in man, intramuscular leucine concentrations are shown to increase when plasma amino acid concentrations are increased by exogenous infusion (Lundholm *et al*, 1987), and to decrease when plasma leucine concentration is decreased by infusion of insulin (Alvestrand *et al*, 1988). The total leucine volume of distribution may be calculated from the sum of leucine and α–ketoisocaproate concentrations, as leucine rapidly and reversibly transaminates to its keto-acid (Matthews *et al*, 1981; Cheng *et al*, 1985; Cheng *et al*, 1987).

During a primed constant infusion of [1–^{13}C]leucine, samples are obtained to determine the ^{13}C enrichment and concentration of plasma α–ketoisocaproate and, in addition, the plasma leucine concentration.

The whole-body leucine rate of appearance is calculated with the following equation (Miles *et al*, 1983):

$$Ra = \frac{(i \bullet E_i) - \left(\frac{P_1 + P_2}{2}\right)\left(\frac{E_{p2} - E_{p1}}{t_2 - t_1}\right)}{\frac{E_{p1} + E_{p2}}{2}} - i$$

Where:

Ra = whole-body leucine rate of appearance (μmol•kg^{-1}•h^{-1}),

i = infusion rate of the [1–^{13}C]leucine tracer (μmol•kg^{-1}•h^{-1}),

E_i = molar enrichment of the [1–^{13}C]leucine tracer (atoms excess),

P_1 and P_2 = whole-body leucine pool sizes at two consecutive time points, and

E_{p1} and E_{p2} = fractional molar ^{13}C enrichments of plasma α–ketoisocaproate (atoms excess) at these time points.

The whole-body leucine pool size (P_1 and P_2) is calculated using the following equation:

$$P = (C_{leu} + C_{KIC}) \bullet 0.6$$

Where:

C_{leu} = plasma leucine concentration (μmol/l), and

C_{KIC} = plasma α–ketoisocaproate concentration (μmol/l).

Units are μmol/kg and the constant 0.6 is derived from the value for total-body water distribution.

The whole-body leucine rate of appearance (Rd) is calculated with the following equation:

$$Rd = Ra - \frac{(P_2 - P_1)}{(t_2 - t_1)}$$

The endogenous component of leucine appearance (protein breakdown) is calculated with the following equation:

$$End\text{-}Ra = Ra - D$$

Where:

End-Ra = endogenous component of whole-body leucine appearance ($\mu mol \bullet kg^{-1} \bullet h^{-1}$), and

D = rate of administration of exogenous leucine or dietary leucine ($\mu mol \bullet kg^{-1} \bullet h^{-1}$).

The non-oxidised component of leucine disappearance (protein synthesis) is calculated as follows:

$$NOLD = Rd - O$$

Where:

NOLD = non-oxidised component of whole-body leucine disappearance ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), and

O = whole-body leucine oxidation ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) calculated as in section 2.3.1.

A number of assumptions underlie the use of these equations. These are the same as the assumptions for leucine modelling under steady state conditions with the following additional assumptions:

a) The plasma concentrations of leucine and α–ketoisocaproate reflect the intracellular concentrations in all cells of the body.

b) The volume of distribution of whole-body free leucine and of α–ketoisocaproate is 0.6 μmol/l.

2.4 Whole-body Protein Turnover Determined with [^{15}N]Phenylalanine: Equations for Steady State Conditions

The ^{15}N enrichment of plasma phenylalanine, during a primed continuous infusion of L–[^{15}N]phenylalanine under plateau conditions, is used to calculate the components of whole-body phenylalanine kinetics. A two pool model is employed with

a) the free pool of phenylalanine in plasma and in cells and b) the protein-bound pool of phenylalanine (Figure 2.3). The equations are (Clarke and Bier, 1982):

$$Q = B + D$$

Where:

Q = the rate of phenylalanine turnover or flux,

B = endogenous phenylalanine appearance or protein breakdown, and

D = dietary intake of phenylalanine.

The units of whole-body phenylalanine kinetics are micromoles of phenylalanine per kilogram total body weight per hour ($\mu mol \bullet kg^{-1} \bullet h^{-1}$).

The plasma phenylalanine flux is calculated with the equation:

$$Q = i \left(\frac{E_i}{E_p} - 1 \right)$$

Where:

i = infusion rate of L–[^{15}N]phenylalanine ($\mu mol \bullet kg^{-1} \bullet h^{-1}$),

E_i = ^{15}N enrichment of the infused L–[^{15}N]phenylalanine (APE), and

E_p = ^{15}N enrichment of plasma phenylalanine (APE) after tracer equilibrium. In the studies reported here the femoral vein was used as sampling site. Ideally with phenylalanine the tracer would have been infused into the left heart and well mixed samples obtained from the right heart (Layman and Wolfe, 1987), a clearly impractical approach for studies in man. By infusing into a vein left heart infusion is approximated and sampling from a vein draining a large vascular bed may have

enabled samples to be obtained which would have been little different from right heart samples.

The first steps in the catabolism of phenylalanine involve hydroxylation of the aromatic ring, producing tyrosine. To calculate phenylalanine entry into protein an estimate of the rate of conversion of phenylalanine to tyrosine is required: this is calculated from simultaneous measurements of phenylalanine flux, tyrosine flux (with an additional tyrosine tracer), and appearance of the infused phenylalanine tracer in plasma tyrosine. In the studies reported here tyrosine flux and phenylalanine catabolism to tyrosine were not measured and, therefore, no estimate of non-catabolized phenylalanine disappearance (i.e. protein synthesis) can be made. Only whole-body total phenylalanine appearance and protein breakdown can be measured with the technique used. The equations of whole-body phenylalanine turnover are only valid where plateaux exist for both the ^{15}N enrichment and the concentration of plasma phenylalanine.

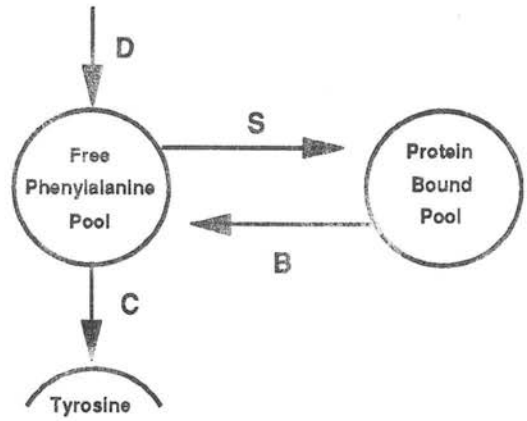

Figure 2.3. Two pool model of protein turnover with phenylalanine; D, diet; S, synthesis; B, breakdown; C, catabolism to tyrosine

There are a number of assumptions underlying the use of these equations; these are:

a) The metabolism of ^{15}N-labelled phenylalanine is the same as that of unlabelled endogenous phenylalanine.

b) There is negligible recycling of tracer after it is incorporated into protein.

c) There are two pools of phenylalanine: a) free, both in the intravascular and intracellular compartments, and b) bound into protein.

d) The degree of dilution of the label (the enrichment of phenylalanine in plasma) is representative of that of intracellular phenylalanine in the precursor pool for protein synthesis. Although this has not been confirmed in man it is shown in animal tissue *in vitro* and *in vivo*, for leucine at least, that leucyl-tRNA is charged with leucine coming predominantly from extracellular fluid or plasma (see section 2.3.1). It is also known that phenylalanine readily enters into cells by the L-system transporter (used by leucine) and, in addition, in liver by the T-system transporter. Neither of these transporters are dependent on hormonal changes (Yudilevich and Boyd, 1987). It, therefore, appears likely that the phenylalanyl–tRNA in man may charge predominantly from plasma, and that tracer studies in man in which plasma phenylalanine enrichment is measured provide some measure of metabolism at an intracellular level. This approach awaits validation.

e) In subjects where phenylalanine was infused parenterally it entered into cells and mixed fully throughout the free phenylalanine pool.

2.5 Anterior tibialis muscle protein synthesis measured from incorporation of [1-^{13}C]leucine

This is an invasive technique in which mixed protein synthetic rate may be determined from samples of muscle obtained by percutaneous muscle biopsy. During a primed continuous infusion of L-[1-^{13}C]leucine a short period of time is allowed to elapse in which equilibration of the tracer occurs throughout plasma and muscle free leucine pools; a plateau of leucine tracer enrichment can then be attained. At this point the tracer labelling of leucyl-tRNA in the protein synthetic precursor pool should also be at plateau; if protein synthesis occurs at a steady rate, [1-^{13}C]leucine incorporation into protein will be constant with respect to time (Figure 1.6). The ^{13}C enrichment of plasma α-ketoisocaproate provides a measure of that of leucyl-tRNA. When biopsies are taken at two time points, during steady-state ^{13}C enrichment of leucine, skeletal muscle protein fractional synthetic rate can be calculated with the following equation (Rennie *et al*, 1982b):

$$k_S = \frac{\Delta E_{muscle}}{E_{KIC}} \bullet \frac{1}{\Delta t} \bullet 100$$

Where:

K_S = muscle protein fractional synthetic rate (%/ h),

ΔE_{muscle} = increase in ^{13}C enrichment of muscle-bound leucine during the time between the two biopsies (APE),

E_{KIC} = mean ^{13}C enrichment in plasma α-ketoisocaproate during the period between the two biopsies (APE), and

Δt = time interval between the two biopsies (h).

This technique has fewer underlying assumptions than these for measuring whole-body protein turnover; the assumptions are:

a) There is no difference in the metabolism of tracer-labelled and endogenous leucine.

b) The ^{13}C enrichment of plasma α–ketoisocaproate is representative of that of leucyl-tRNA in the precursor pool for protein synthesis (see section 2.3.1).

2.6 Arteriovenous Phenylalanine Exchange by Leg Tissues Determined with L–[^{15}N]Phenylalanine

This technique may be used to measure arteriovenous uptake and release of phenylalanine across the leg (Gelfand and Barrett, 1987; Thompson *et al*, 1989), which predominantly contains skeletal muscle (Häggmark *et al*, 1978). In skeletal muscle the only metabolic fates of phenylalanine are incorporation into protein and release from protein; catabolism of phenylalanine to tyrosine occurs predominantly in liver and does not occur in muscle. Uptake and release of phenylalanine by the leg are, therefore, indices of protein synthesis and breakdown respectively.

The ^{15}N enrichment of arterial and venous plasma phenylalanine, during a primed continuous infusion of [^{15}N]phenylalanine, and plasma concentrations may be used to calculate phenylalanine exchange by leg tissues from the degree of dilution of

the tracer in the venous outflow in comparison with the arterial inflow. The following equations (simplified from these of Cheng *et al*, 1985) may be used (Gelfand and Barrett, 1987; Thompson *et al*, 1989):

$$Bkdn = \left(\frac{E_{A\text{-}phe}}{E_{V\text{-}phe}} - 1 \right) \bullet C_{A\text{-}phe} \bullet BF$$

Where:

Bkdn = release of phenylalanine (protein breakdown) across the leg in nmol of phenylalanine per 100 g of leg tissue per minute ($nmol \bullet 100\ g^{-1} \bullet min^{-1}$),

$E_{A\text{-}phe}$ = ^{15}N enrichment of arterial plasma phenylalanine (APE),

$E_{V\text{-}phe}$ = ^{15}N enrichment of venous plasma phenylalanine (APE),

$C_{A\text{-}phe}$ = arterial plasma concentration of phenylalanine in nmol/ml or µmol/l, and

BF = blood flow of the leg in ml per 100 g leg per minute ($ml \bullet 100\ g^{-1} \bullet min^{-1}$).

The net arteriovenous balance or the efflux of phenylalanine, a measure of protein balance, may be calculated with the following equation:

$$Bal = (C_{A\text{-}phe} - C_{V\text{-}phe}) \bullet BF$$

Where:

Bal = arteriovenous balance of phenylalanine across the leg ($nmol \bullet 100\ g^{-1} \bullet min^{-1}$), and

$C_{A\text{-}phe}$ and $C_{V\text{-}phe}$ = arterial and venous plasma phenylalanine concentrations (µmol/l).

The term *flux* should be avoided and the term balance is preferred. The former term was coined before the application of tracer techniques to the investigation of leg amino acid metabolism. The results with tracer techniques show that the flux of "flow" of amino acids is much greater than their respective net balances.

The arteriovenous uptake of phenylalanine or protein synthesis is calculated with the equation:

$$Syn = Bal = Bkdn$$

Where:

Syn = arteriovenous uptake of phenylalanine ($nmol \cdot 100\ g^{-1} \cdot min^{-1}$).

These equations are only applicable for a steady-state of concentration and ^{15}N enrichment of plasma phenylalanine.

There are a number of assumptions which are integral to use of these equations; these are:

a) There is no difference between the metabolism of ^{15}N-labelled and endogenous phenylalanine.

b) There is negligible recycling of tracer after incorporation into protein.

c) Rapid exchange of phenylalanine occurs between plasma and muscle and, therefore, the ^{15}N enrichment of venous phenylalanine represents that entering protein synthesis within muscle, i.e. that of muscle phenylalanyl–tRNA.

d) The concentration of phenylalanine in plasma is the same as that of free phenylalanine within muscle.

e) When steady-state conditions are changed by a therapeutic action, complete exchange of phenylalanine occurs in the intramuscular free pool before further measurements are made.

f) The blood cells exchange amino acids rapidly and are involved in the across-limb amino acid exchange. Pilot studies in our laboratory suggest that this is the case

but exchange of tracer by the blood elements may be slower than that of plasma (K. Smith, M. J. Rennie, personal communication).

g) The arterial blood flow is the same as the venous blood flow.

2.7 Arteriovenous Leucine Exchange by Leg Tissues Determined with [1-^{13}C]Leucine

This technique may be used to measure uptake and release of leucine, that of its keto-acid α-ketoisocaproate, and release of carbon dioxide from oxidation of α-ketoisocaproate by leg tissues (Cheng *et al*, 1985). Leucine may undergo equivalent metabolism in leg tissues as in the whole-body: therefore, leucine may undergo reversible transamination to α-ketoisocaproate or may be incorporated into protein (Figure 2.1). The α-ketoisocaproate may be irreversibly decarboxylated to produce isovaleryl-CoA with loss of the ^{13}C label as $^{13}CO_2$ (Figure 2.1). In arteriovenous exchange studies these metabolic fates of leucine must be accounted for if accurate measures are to be calculated for leucine incorporation into and release from protein.

^{13}C enrichments and concentrations of arterial and venous plasma leucine, plasma α-ketoisocaproate and of blood carbon dioxide are measured in samples obtained during plateau conditions of a primed continuous infusion of L-[1-^{13}C]leucine. Oxidation of α-ketoisocaproate by the leg may be calculated from the equation (Cheng *et al*, 1985):

$$\text{Oxid} = \frac{(C_{V\text{-}CO_2} \bullet E_{V\text{-}CO_2}) - (C_{A\text{-}CO_2} \bullet E_{A\text{-}CO_2})}{E_{V\text{-}KIC}} \bullet BF \bullet 1000$$

Where:

Oxid = oxidation of α–ketoisocaproate (nmol•100 g^{-1}•min^{-1}),

$C_{V\text{-}CO_2}$ and $C_{A\text{-}CO_2}$ = concentration of carbon dioxide in venous and arterial blood (mmol/l),

$E_{V\text{-}CO_2}$ and $E_{A\text{-}CO_2}$ = ^{13}C enrichment of carbon dioxide in venous and arterial blood (APE),

$E_{V\text{-}KIC}$ = ^{13}C enrichment of venous plasma α–ketoisocaproate (APE), and

BF = blood flow (ml•100 g^{-1}•min^{-1}).

The constant 1000 changes µmol•100 g^{-1}•min^{-1} to nmol•100 g^{-1}•min^{-1}.

The sum of the plasma concentrations of leucine and α–ketoisocaproate is used in the calculations of leucine exchange by the leg. Termed the total leucine carbon (TLC), this (for the arterial value) may be calculated as follows:

$$C_{A\text{-}TLC} = C_{A\text{-}leu} + C_{A\text{-}KIC}$$

Where:

$C_{A\text{-}TLC}$ = combined arterial plasma concentrations of leucine and α–ketoisocaproate, the total leucine carbon (µmol/l),

$C_{A\text{-}leu}$ = arterial plasma concentration of leucine (µmol/l), and

$C_{A\text{-}KIC}$ = arterial plasma concentration of α–ketoisocaproate.

Equivalent equations may be used to calculate the venous concentration of total leucine carbon.

The combined ^{13}C enrichment of arterial plasma leucine and plasma α–ketoisocaproate (the arterial ^{13}C enrichment of total leucine carbon) may be calculated with the equation:

$$E_{A\text{-}TLC} = \frac{(E_{A\text{-}leu} \bullet C_{A\text{-}leu}) + (E_{A\text{-}KIC} \bullet C_{A\text{-}KIC})}{C_{A\text{-}TLC}}$$

Where:

$E_{A\text{-}TLC}$ = arterial ^{13}C enrichment of plasma total leucine carbon (APE),

$E_{A\text{-}leu}$ = arterial ^{13}C enrichment of plasma leucine (APE), and

$E_{A\text{-}KIC}$ = arterial ^{13}C enrichment of plasma α–ketoisocaproate (APE).

The venous ^{13}C enrichment of plasma total leucine carbon may be calculated with analogous equations.

The net balance of leucine incorporated into protein may be calculated with the equation:

$$Bal = [(C_{A\text{-}TLC} - C_{V\text{-}TLC}) \bullet BF] - Oxid$$

Where:

Bal = net leucine balance between protein synthesis and protein breakdown, (i.e. net protein synthesis) in nmol of leucine•100 g^{-1}•min^{-1}.

The rate of release of leucine from protein (i.e. protein breakdown) may be calculated with the equation:

$$Bkdn = \left(\frac{E_{A\text{-}TLC}}{E_{V\text{-}TLC}} - 1 \right) \bullet BF \bullet C_{A\text{-}TLC} - Oxid$$

Where:

Bkdn = total leucine release from protein (nmol•100 g^{-1}•min^{-1}).

The rate of total entry of leucine into protein (i.e. protein synthesis) may be calculated with the following equation:

$$Syn = Bal + Bkdn$$

Where:

Syn = protein synthesis ($nmol \cdot 100\ g^{-1} \cdot min^{-1}$).

There are a number of assumptions which underlie these equations; these are:

a) There are no difference between the metabolism of [1–^{13}C]-labelled and endogenous leucine and α–ketoisocaproate.

b) There is negligible recycling of tracer after it is incorporated into protein.

c) Rapid exchange of leucine and α–ketoisocaproate occurs between plasma and muscle and the ^{13}C enrichment of venous leucine and α–ketoisocaproate is representative of that entering protein synthesis within muscle, i.e. that of muscle leucyl-tRNA.

d) The concentration of leucine and α–ketoisocaproate in plasma is the same as that free in intramuscular water.

e) Where steady-state conditions are changed by a therapeutic action an complete exchange of leucine and α–ketoisocaproate occurs in the intramuscular free pools before further measurements are made.

f) Venous plasma α–ketoisocaproate ^{13}C enrichment is representative of that of α–ketoisocaproate undergoing oxidation.

g) Blood cells exchange amino acids rapidly and are involved in the across-limb amino acid exchange.

h) The arterial blood flow is the same as the venous blood flow.

2.8 Myofibrillar Protein Breakdown Determined by Release of 3-Methylhistidine from the Leg

3-Methylhistidine (i.e. N^{τ}-methylhistidine) is an amino acid produced by post-translational methylation at the N^{τ} position of histidine-73 of the actin chain (Vanderkerckhove and Webster, 1984). When released by the breakdown of myofibrillar proteins, 3-methylhistidine is not reincorporated into protein by protein synthesis and does not undergo intermediary metabolism (Young *et al*, 1973). 3-Methylhistidine, or its hepatically acylated derivative, is excreted and provides a quantitative marker of myofibrillar protein breakdown.

3-Methylhistidine is a constituent of actin (Asatoor and Armstrong, 1967) and the heavy chain of myosin in white muscle (Johnson *et al*, 1967), proteins which together constitute some 50% of muscle protein. Release of 3-methylhistidine, therefore, provides a specific index of breakdown of these myofibrillar proteins. It follows that factors modulating the breakdown of actin and myosin will induce changes in 3-methylhistidine release. In contrast, factors modulating changes in the rate of breakdown of mixed non-myofibrillar proteins may not cause any change in 3-methylhistidine release. Therefore, apparently conflicting results may be obtained from studies comparing changes in protein breakdown measured using 3-methylhistidine and phenylalanine release (Smith and Sugden, 1986).

The release of 3-methylhistidine in urine has been widely advocated and used as an index of skeletal muscle protein breakdown *in vivo* (Ballard and Tomas, 1983). Objections to such use (Rennie and Millward, 1983) are based on the existence of 3-methylhistidine in non-muscle protein pools which turnover rapidly and contribute

substantially to the total urine release of 3–methylhistidine (Clark and Spudich, 1977; Young and Munro, 1978; Emery *et al*, 1986). For the rat, estimates of the source of total 3–methylhistidine release from skeletal muscle, skin and gastrointestinal muscle are 25%, 7% and 10% respectively; the source of more than half of the total urine excretion of 3–methylhistidine was not identified (Millward *et al*, 1980c). For healthy man such information is not available. However, in man during sepsis about 80% of excreted 3–methylhistidine derives from skeletal muscle with approximately 20% from non-muscle sources (Sjölin *et al*, 1989). In studies of human protein metabolism seemingly directly conflicting results are reported with regard to the changes which may occur in urine excretion and leg efflux of 3–methylhistidine (Rennie *et al*, 1983). It is, therefore, likely that urine excretion of 3–methylhistidine is not adequately specific for monitoring human skeletal muscle protein breakdown *in vivo* and only 3–methylhistidine efflux from the leg has been employed as an index of skeletal muscle breakdown in the studies reported here. The major difficulty with the leg arteriovenous exchange technique is that the plasma concentration of 3–methylhistidine is very low (2–6 μmol/l) and can only be measured with sophisticated techniques. Therefore, in the present studies results are only available from the group of healthy subjects where amino acid analysis was by automated ion exchange chromatography with fluorometric detection.

The 3–methylhistidine efflux was calculated with the following equation:

$$Bal = (C_{A\text{-}3\text{-}mehis} - C_{V\text{-}3\text{-}mehis}) \bullet BF$$

Where:

$C_{A\text{-}3\text{-}mehis}$ and $C_{V\text{-}3\text{-}mehis}$ = plasma concentrations of 3–methylhistidine (μmol/l), and

BF = blood flow ($ml \cdot 100\ g^{-1} \cdot min^{-1}$).

Units are nmol of 3-methylhistidine per 100 g per minute ($nmol \cdot 100\ g^{-1} \cdot min^{-1}$).

Chapter 3. GENERAL METHODOLOGY

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CLINICAL METHODOLOGY

3.1 Subjects

Healthy volunteers were recruited from the staff of the University of Dundee and Ninewells Hospital and Medical School, Dundee, from the students of the University of Dundee and from the general public. Type 1 diabetic subjects were recruited from the diabetic outpatient clinic at Ninewells Hospital and Medical School. The healthy subjects had no acute or chronic illness and were not taking any medication. The diabetic subjects were all established on insulin treatment, had stable insulin-dependent diabetes and were free from major diabetic complications: their only treatment was insulin. In all subjects biochemical tests of renal, hepatic and thyroid function were normal and the diabetic subjects did not have proteinuria on repeated testing. All subjects had stable body weights prior to study and were consuming weight-maintaining diets. The diabetic subjects were prescribed diets in which carbohydrate supplied over 50% and fat 35 to 40% of the energy contents. The carbohydrate component of the diet was not highly refined and had a high fibre content.

Each subject was given a full verbal and written explanation of the procedures to be undertaken and their risks. Each subject gave written consent before participation in the study. The research protocols were approved by the Tayside Health Board Committee on Medical Ethics.

3.2 Tracers

The stable isotope labelled amino acids L–[1–^{13}C]leucine (99% ^{13}C) and L–[^{15}N]phenylalanine (99% ^{15}N), and sodium [^{13}C]bicarbonate (99% ^{13}C) were purchased from Tracer Technologies Incorporated, Newton, MA, U.S.A. L–[*phenyl*–$^{2}H_5$]phenylalanine (98% $^{2}H_5$) was obtained from Cambridge Isotope Laboratories, Woburn, MA, U.S.A.. Immediately prior to administration the tracers were mixed in sterile pyrogen-free sodium chloride solution (150 mmol/l), using an aseptic technique, and the tracers were sterilized by passage through 0.22 μm filters (Millipore SA, Molsheim, France). The tracer infusates were diluted to a total volume of 500 ml in glass bottles and were infused via a second 0.22 μm filter.

3.3 Materials

Neutral human insulin (Humulin S, Eli Lilly and Company Ltd, Basingstoke, U.K.) was infused during the euglycaemic insulin clamp studies. Commercial mixed amino acid solutions (Synthamin 9 and Synthamin 14) were obtained from Travenol Laboratories Ltd, Thetford, U.K.; their composition is shown in Table 3.1. During the euglycaemic insulin clamp studies potato-starch derived glucose was infused: this had a low natural abundance of ^{13}C (–20.180 $\delta^{13}C_{PDB}‰$) as opposed to regular maize-derived glucose (–9.85 $\delta^{13}C_{PDB}‰$) (Lacroix *et al*, 1973; Schoeller *et al*, 1980; Scrimgeour *et al*, 1988b). The potato-derived glucose (Tunnel Refineries Ltd., Greenwich, U.K.) was dissolved (1 mol/l) in distilled water, passed through 0.22 μm filters, autoclaved and demonstrated to be

sterile, low in particulate matter and pyrogen free, before infusion into the subjects.

Table 3.1. Composition of Synthamin Amino Acid Solution

Amino acid	Synthamin 9 (g/l)	Synthamin 14 (g/l)
Alanine	11.38	17.60
Arginine	6.32	9.78
Glycine	5.66	8.76
Histidine	2.64	4.08
Isoleucine	3.30	5.10
Leucine	4.02	6.20
Lysine	3.19	4.93
Methionine	2.20	3.40
Phenylalanine	3.08	4.76
Proline	3.74	5.78
Serine	2.75	4.25
Threonine	2.31	3.57
Tryptophan	0.99	1.53
Tyrosine	0.22	0.34
Valine	3.19	4.93
Total L-Amino Acids	55.0	85.0
Total Nitrogen	9.1	14.0

3.4 Experimental Environment

All subjects were admitted to the Department of Medicine, Ninewells Hospital and Medical School at 08.30 h on the day of study. Their weight, height and body fat composition (from skinfold thickness at four sites, Durnin and Womersley, 1974), were determined and thereafter the subjects remained semirecumbent throughout the study. The study room was maintained at a thermoneutral temperature (25 to 27°C).

3.5 Blood Sampling

Arterialized venous blood samples were taken from a 1.7 mm outer-diameter cannula (Venflon, Viggo AB, Helsingborg, Sweden) placed retrogradely in a dorsal hand vein, and maintained patent by a slow infusion of sodium chloride solution (150 mmol/l). Before sampling the hand was warmed for a minimum of 15 min by insertion into a thermostatically controlled chamber with a circulating air temperature of 75 to 80°C (Abumrad *et al*, 1981). A requirement of the whole-body kinetic technique is the need to obtain mixed blood which is representative of that in all tissues, i.e. arterial blood, and the arteriovenous exchange technique also places a requirement for arterial samples. Arterial cannulation poses unacceptable risks to healthy subjects (Hall, 1971), and was not used in the present studies. The arterialized venous "hot-box" technique that was utilized has been validated for obtaining arterial samples for blood gases and lactate (Forster *et al*, 1972), glucose and alanine (Abumrad *et al*, 1981), and ketone bodies (Sonnenberg and Keller, 1982).

A second 1.7 mm outer-diameter cannula was placed in a vein of the contralateral forearm for infusion of amino acids, tracers, insulin and substrates. In the arteriovenous exchange studies a third 1.7 mm. outer-diameter cannula (5½" Abbocath-T, Abbott Hospitals Incline, North Chicago, IL, U.S.A.) was placed in either common femoral vein for sampling the mixed venous outflow from the leg. To insert this longer sampling cannula, a shorter cannula (45 mm long by 1.7 mm outer-diameter, Argyle Medicut, Sherwood Medical, Tullamore, Ireland) was first used to cannulate the vein, through which an 18 gauge flexible wire was introduced. The Abbocath-T cannula was then inserted over the guide-wire into the common femoral vein. Local anaesthetic (lignocaine 200 g/l) was infiltrated before all cannulations.

A sphygmomanometer cuff placed around the ankle was inflated to a pressure of 200 mmHg for 60 sec before femoral blood sampling to occlude the venous drainage of the foot. Blood was removed from the femoral vein canula at a rate of only 1 ml/sec to avoid disturbing the blood flow and tracer equilibrium of the leg. The femoral site allows sampling of mixed venous blood from the leg. In sedentary subjects the thigh is composed of about 82% muscle (Häggmark *et al*, 1978) and skin accounts for only a small proportion but does, at least in rats, have a relatively fast protein turnover rate in comparison to muscle. In rats, the muscle protein turnover rate is about 6 to 9% per day and in skin the rate (20 to 35% per day) is about 3 to 4 times faster (Chikenji *et al*, 1983). In addition in man bone accounts for about 10% of leg volume: this tissue has a very slow turnover rate and bone blood flow was not recorded by the technique used in these studies. The remaining proportion of leg, adipose tissue, has a low unit blood flow and

contributes to phenylalanine metabolism to a negligible extent. This suggests that muscle protein turnover predominantly accounts for the protein turnover of the human leg.

Table 3.2. Additives used in Blood Sample Tubes

Analysis	Additive	Quantity
Leucine Enrichment	Li Hep	
Phenylalanine Enrichment	Li Hep	
α-ketoisocaproate Enrichment	Li Hep	
α-ketoisocaproate Conc.	Li Hep	
Amino acid Conc.	Li Hep	
Glucose	Fluoride Oxalate	
D-(-)-3-hydroxybutyrate Conc.	Perchloric acid	1:1 v/v
Lactate	Perchloric acid	2:1 v/v
Insulin	Li Hep	
Cortisol	Li Hep	
C-Peptide	Li Hep	
Glucagon	EDTA-Aprotinin	500 KIU/ml
Insulin-like Growth Factor 1	EDTA	
Packed Cell Volume	Li Hep	
Blood Gas Conc.	Li Hep (syringe)	
Blood CO_2 Enrichment & Conc.	Orthophosphoric acid	

Abbreviations: Li Hep, lithium heparin; EDTA, ethylenediaminetetra acetic acid; KIU, kallikrein inhibitor units.

3.5.1 Blood Sample Handling

The blood samples were added to tubes which contained additives appropriate for subsequent biochemical assays (Table 3.2). All samples were stored on ice prior to separation of plasma in a refrigerated centrifuge (4°C) at 1700 times gravity for 20 min. Samples were stored at -20°C until analysis with the exception of the samples for glucagon and lactate which were stored at -80 or -196°C. To prepare plasma for amino acid concentration analysis, the plasma was deproteinised with sulphosalicylic acid (100 g/l, 1:4 v:v) containing norvaline internal standard (64 μmol/l), incubated for 30 min at 4°C to precipitate β-lipoproteins, passed through 0.45 μm filters before storage at -20°C. The perchloric acid extracts of blood for D-(-)-3-hydroxybutyrate analysis were neutralised by the addition of an alkaline solution containing 2 mol/l KOH, 0.4 mol/l KCl and 0.4 mol/l imidazole before storage frozen. Free insulin concentration was determined in the insulin treated diabetic subjects after removal of antibody-bound insulin with the following method (Rudkowski and Antony, 1986): blood was centrifuged immediately after sampling and the plasma was mixed with an equal volume of a solution of polyethylene glycol 6000 (250 g/l, biochemistry grade, in phosphate buffer 0.05 mol/l, pH 7.4), centrifuged again and the supernatant stored at -20°C before insulin radioimmunoassay. To treat and store blood for subsequent determination of whole blood carbon dioxide concentration and ^{13}C enrichment, the blood (1 ml) was added to 20 ml glass tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, U.S.A.) immediately on sampling: before use these tubes were prepared by adding 1 ml of a mixture of

orthophosphoric acid (6 mol/l) and silicone antifoam (10% by volume) and the tubes were degassed to a pressure of 0.4 mbar and sealed (Read *et al*, 1984).

3.6 Breath Sampling and Indirect Calorimetry

Before tracer administration and at intervals throughout the studies expired breath was collected into 2 l latex bags and an aliquot was transferred into 20 ml evacuated glass tubes (Vacutainer) for storage and subsequent determination of ^{13}C enrichment in carbon dioxide, as detailed below. Total carbon dioxide production and oxygen consumption was determined with a microcomputer-controlled, ventilated-hood, indirect calorimeter (Illingworth *et al*, 1986). This continually extracted air at a rate of 45 l/min from a plastic hood, fitted over the patients head, and passed this via a calcium chloride-packed drying column to an infrared carbon dioxide detector (SS-200, Analytical Development Company Ltd., Hoddesdon, U.K.) and a paramagnetic oxygen detector (Servomex 540A, Taylor Instrument Ltd., Crowborough, U.K.). The analogue detector outputs were connected through an analogue-to-digital signal converter to a microcomputer. Barometric pressure was monitored continuously with a digital meter and the microcomputer was programmed with the ambient air temperature. Carbon dioxide production and oxygen consumption were calculated each minute and results were expressed in l/min at standard temperature and pressure. Before and after each calorimetry session the gas detectors were calibrated with standard gas mixtures: for the oxygen meter these were nitrogen, for zero calibration, and room air for 20.94% O_2; for the carbon dioxide meter nitrogen was used and a gas mixture containing 0.8%

carbon dioxide. From the oxygen uptake and carbon dioxide output resting energy expenditure was calculated with Weir's formula (Weir, 1949).

3.7 Muscle Biopsy Technique

Anterior tibial muscle was biopsied using 6.5 mm Tilley Henckel ethmoid punches (S. Murray and Co., Sheffield, U.K.) via a 10 mm long skin incision made after 5 ml of 20 g/l lignocaine local anaesthetic was infiltrated superficially to the fascia (Dietrichson *et al*, 1987). At each time point one to three biopsies were obtained providing 100 to 300 mg wet weight of muscle. The biopsies were from the fullest part of the shin and were taken 10 mm deep to the fascia. After each biopsy firm pressure was applied for 10 to 15 min over the site, and the skin incision was held closed with surgical tape. A second biopsy was taken from either the same leg, at a distance of 40 mm to the first biopsy, or from the contralateral leg. A third biopsy was taken from the leg contralateral to that first biopsied. The muscle samples were immediately frozen in liquid nitrogen and stored at -196°C until analysis.

3.8 Leg Blood Flow

Leg blood flow was measured by mercury-silastic strain-gauge plethysmography (Whitney, 1953; Needham, 1972; Englund *et al*, 1972; Sumner, 1985). The technique is shown to give reproducible results (Roberts *et al*, 1986) and to show appropriate blood flow changes resulting from exercise (Hughson, 1988). Occlusion of the venous return from the leg appears to result in underestimation of leg blood flow (Hiatt *et al*, 1989). With the subject

semirecumbent their heel was raised 200 mm from the bed and the maximal circumference of their calf was measured. A strain-gauge of appropriate length was selected and a baseline signal obtained by adjusting the balance resistance of a Wheatstone Bridge when a 20 g weight was suspended on the gauge to tension it. The gauge output was displayed on a chart recorder with a range setting of 10 mV per 200 mm and paper speed of 2 mm/sec. The gauge was then placed round the calf and the gauge was tensioned, by adjustment of a lengthening screw, to produce the same electrical deflection as that produced by the 20 g weight. The system was then repeatedly calibrated by adjusting the calibration screw of the gauge four turns (a distance of 1.818 mm) which produced a deflection on the pen recorder. When the venous return from the leg was transiently occluded, by inflation of a large thigh cuff to a pressure of 60 mmHg, the arterial input remained unaltered and a linear increase occurred in both leg volume and diameter, from which blood flow could be measured by comparison with the calibration deflections.

A second cuff around the ankle was inflated above arterial blood pressure (200 mmHg) to exclude blood flow from the foot for 2 min prior to blood flow measurements. Then the thigh cuff was automatically inflated to a pressure of 60 mmHg for 14 sec, during which time blood flow was recorded, and the cuff was then deflated for 6 sec to allow venous drainage from the leg. The procedure was repeated a minimum of three times at each time point.

3.8.1 Equation for Calculating Leg Blood Flow:

$$\text{Blood Flow} = \frac{S \bullet 200}{C \bullet d}$$

Where:

S = Slope of trace • 120 (mm/min),

C = Calibration height on chart recorder equivalent to a 1 mm change in gauge circumference (mm), and

d = calf circumference at the level of the gauge (mm).

The constant 200 relates the rate of change in gauge length to blood flow per unit weight (100 g) of leg tissue. Units are ml per 100 g of leg tissue per min ($ml \bullet 100\ g^{-1} \bullet min^{-1}$).

3.8.2 Strain-Gauge Construction

The mercury-silastic gauges were constructed from fine bore silastic tubing (0.5 mm inner-diameter, 0.5 mm wall thickness), filled with a column of mercury and plugged with copper at each end to complete the electrical circuit and to anchor the ends of the silastic tubing to the gauge mounts (Needham, 1972). A Wheatstone Bridge circuit was employed, with adjustable rheostats, and this was connected to the pen recorder to obtain traces of the changes in gauge length and hence electrical resistance.

3.9.1 Tracer Infusions

At the start of tracer infusion doses of L–[1–^{13}C]leucine (0.9 mg/kg) and sodium [^{13}C]bicarbonate (either 0.16 or 0.24 mg/kg) were administered to prime their respective body pools (Allsop *et al*, 1978; Matthews *et al*, 1980). A continuous infusion of L–[1–^{13}C]leucine at a rate of 1 mg•kg^{-1}•h^{-1} was administered throughout the studies. This was diluted to a volume of 500 ml in sodium chloride (150 mmol/l), and was delivered at a rate of either 77 or 56 ml/h with IMED 928 volumetric infusion pumps (IMED Ltd, Abington, U.K.). In the arteriovenous exchange studies priming doses of L–[^{15}N]phenylalanine (0.45 mg/kg) were administered, followed by a continuous infusion (0.5 mg•kg^{-1}•h^{-1}) throughout the studies.

3.9.2 Insulin Infusion

Insulin was diluted immediately prior to use (0.5 units/ml) in sodium chloride (150 mmol/l) containing 5% blood (by volume) to minimise adhesion of the insulin to the plastic infusion syringe and tubing. An exponentially-decreasing priming dose was administered over 10 min followed by a continuous infusion at 0.29 nmol•m^{-2}•min^{-1} (40 m–units•m^{-2}•min^{-1}) (DeFronzo *et al*, 1979). The rate of the insulin infusion was controlled with a Treonic IP3 digital syringe pump (Vickers Medical, Basingstoke, U.K.).

3.9.3 Glucose and Mixed Amino Acid Infusions

In the hyperinsulinaemia studies with diabetic subjects glucose was infused when the plasma glucose concentration fell to 5 mmol/l, at a sufficient rate to

maintain a stable plasma glucose concentration of 5 mmol/l. In the healthy subjects studied with insulin infusions the plasma glucose was allowed to fall by 0.3-0.5 mmol/l before glucose was infused: this plasma glucose concentration, 0.3-0.5 mmol/l lower than the subjects fasting glucose concentration, was maintained during the euglycaemic clamp to minimise endogenous secretion of insulin by the subject. Priming doses of mixed amino acid solutions were infused over the first 10 min of the mixed amino acid infusions: these priming dose provided the same dose of mixed amino acids as the amount infused during each hour of the subsequent continuous infusion. This protocol produced steady-state conditions for both the plasma leucine concentration and the ^{13}C enrichments of leucine and α-ketoisocaproate about 30 min after commencement of a mixed amino acid infusion. The solutions of glucose and amino acids were infused and controlled with separate IMED 928 volumetric infusion pumps.

3.10 Protocols of Study

In all of investigations described the subjects consumed a weight-maintaining diet during the week before study. They took their evening meal between 5-30 and 7 pm, on the evening prior to the study. Thereafter they consumed no food by mouth until completion of the study on the following day; only water was given.

Two separate study protocols were used with the muscle biopsy technique to assess leucine incorporation into muscle and whole-body leucine turnover:

3.10.1 Protocol a

This was used to assess the effects of mixed amino acid infusion alone on whole-body leucine turnover and anterior tibialis muscle protein synthesis in healthy postabsorptive men (Figure 3.1). Mean enrichment values during the periods 90-270 and 330-510 min were used as the basis of calculations.

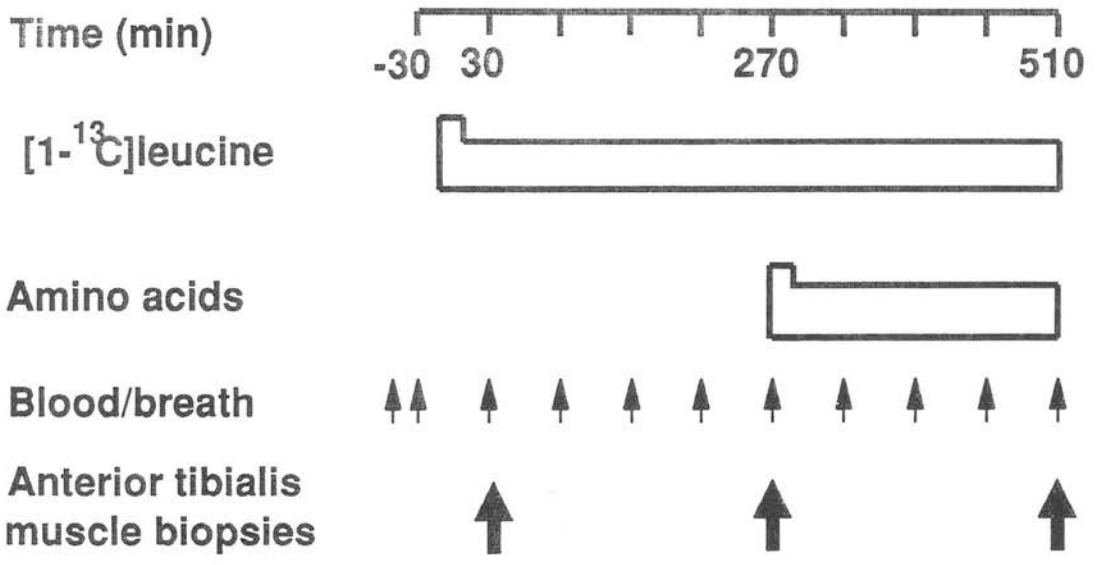

Figure 3.1. Protocol a, The influence of amino acids on leucine incorporation into anterior tibialis muscle

3.10.2 Protocol b

This was used to assess the acute effects of insulin infusion on protein turnover in insulin withdrawn type 1 diabetic subjects. Mixed amino acids were continuously infused to maintain amino acid availability. Whole-body leucine metabolism and anterior tibialis muscle protein synthesis were assessed (Figure 3.2).

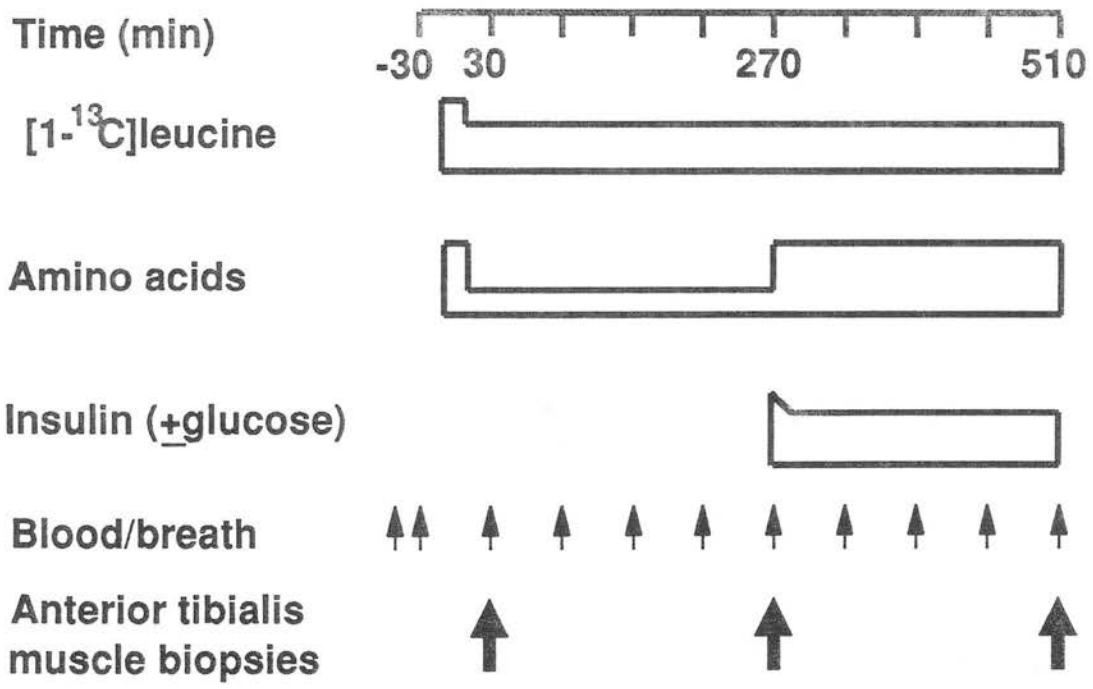

Figure 3.2. Protocol b, The effect of insulin with amino acids on incorporation of leucine into anterior tibialis muscle

Two separate protocols were used to assess leg arteriovenous exchange of phenylalanine and leucine:

3.10.3 Protocol c

This protocol was used to assess the effects of mixed amino acid infusion alone on skeletal muscle protein turnover and whole-body leucine and phenylalanine kinetics in healthy postabsorptive subjects (Figure 3.3). Comparisons were based on mean values during the periods 135-180 and 315-360 min.

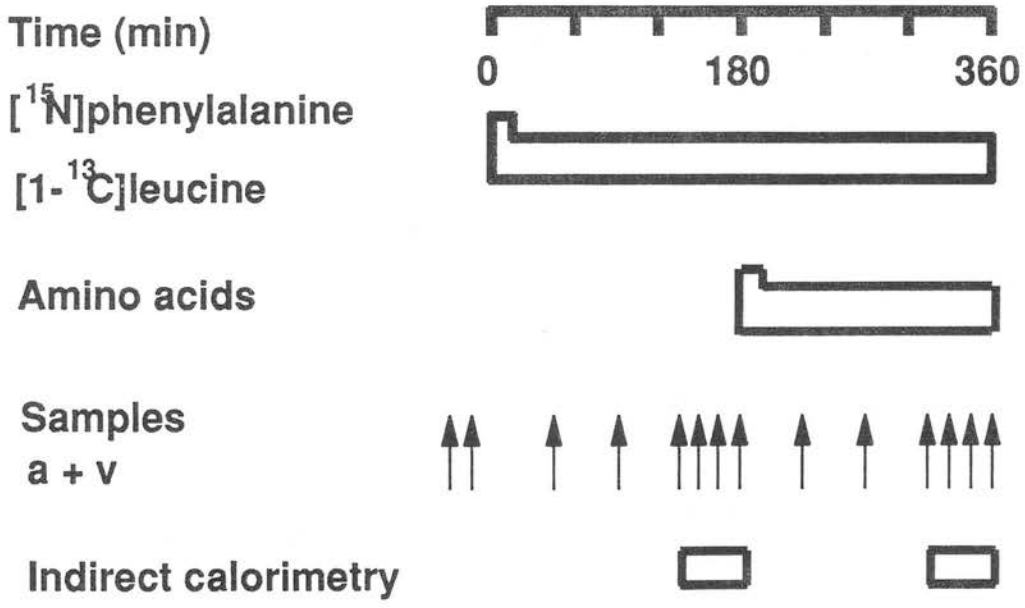

Figure 3.3. Protocol c, The effect of amino acid infusion on leg exchange of phenylalanine and leucine

3.10.4 Protocol d

This was used to assess the acute effects of insulin, with and without mixed amino acid infusion, on skeletal muscle protein turnover and whole-body leucine and phenylalanine kinetics in both healthy subjects and type 1 diabetic subjects (Figure 3.4). Comparisons were based on mean values during the periods 135-180 and 315-360 min.

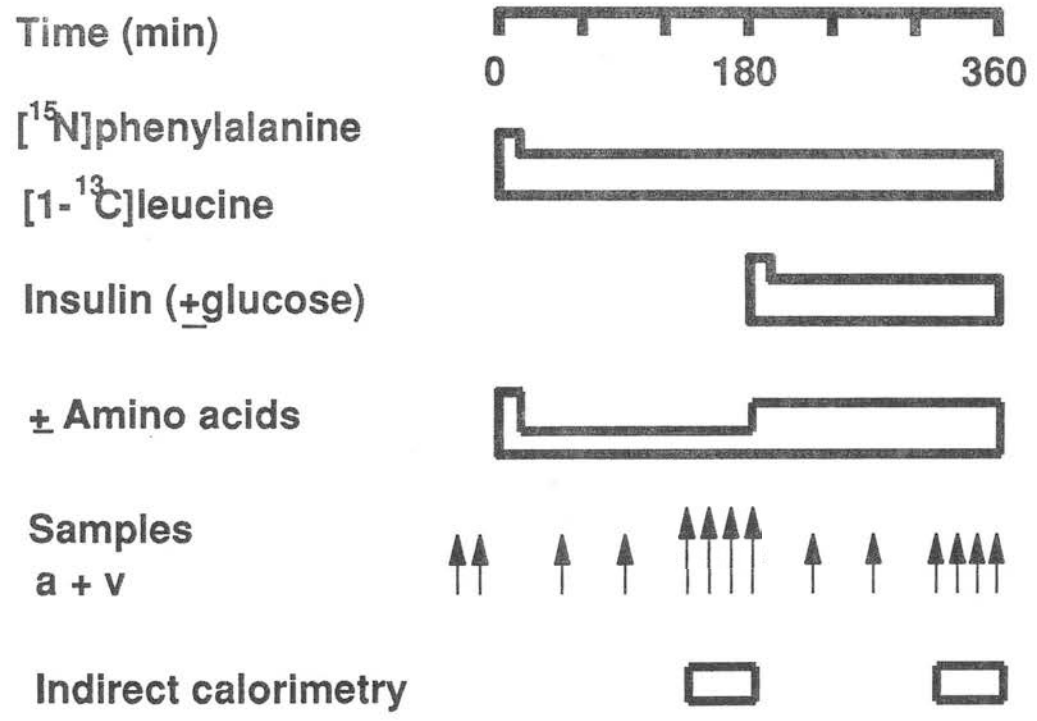

Figure 3.4. Protocol d, The effect of insulin, with and without amino acid infusion, on leg exchange of phenylalanine and leucine

LABORATORY ANALYSIS

3.11 Gas Chromatography Mass Spectrometry

Gas chromatography-mass spectrometry was carried out on either a Finnigan 1020 instrument (Finnigan MAT Ltd, Hemel Hempstead, U.K.) or on a Hewlett-Packard 5971A MSD instrument (Hewlett-Packard Ltd, Cheadle Heath, U.K.). These mass spectrometers were fitted with 20 m and 12 m by 0.2 mm inner diameter OV-1 WCOT chemically bonded fused silica columns (Pierce UK Ltd, Cambridge, U.K.) respectively. Gas chromatography oven temperature was programmed to increase from 140 to 270°C at 20°C/min after a 1 min lag. The injector temperature was 240°C and injection, in the splitless mode, using decane as solvent was complete in 30 s. The carrier gas was helium at 50 kPa. The instruments were operated in the electron-impact mode with an ionization energy of 70 eV.

3.11.1 ^{13}C Analysis of Plasma Leucine

1-^{13}C Enrichment of leucine in plasma was determined after extraction of amino acids and preparation of *tert*-butyldimethylsilyl derivatives by reaction with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (Regis Chemical Company, Morton Grove, IL, U.S.A.) (Mawhinney *et al,* 1986) (Table 3.3). The [1–^{13}C] enrichment in leucine was determined by monitoring ion currents m/z 302 and 303. The retention time for this derivative of leucine was 3.7 to 3.8 min.

3.11.2 ^{15}N Analysis of Plasma Phenylalanine

The preparation of plasma phenylalanine samples for ^{15}N enrichment analysis by gas chromatography-mass spectrometry was identical to that used for leucine (Table 3.3). The same ion currents were monitored. The retention times for the phenylalanine derivative were in the range of 5.6 to 5.8 min (longer than these for leucine). The analysis of leucine and phenylalanine was normally carried out on the same sample.

3.11.3 ^{13}C Analysis of Intramuscular Free Leucine

The ^{13}C enrichment of intramuscular leucine was determined in neutralized perchloric acid (0.5 mol/l) extracts of ground, freeze-dried muscle. The samples were purified using cationic ion exchange-chromatography (Table 3.4). *t*-Butyldimethylsilyl derivatives were prepared and gas chromatography-mass spectrometry was carried out a minimum of two times on each sample. When calculating the ^{13}C enrichment for the intramuscular leucine a correction was made to the measured value on the basis that 13% of the water content of the samples, by weight, was extracellular (Bergström *et al*, 1974).

3.11.4 ^{13}C Analysis of Plasma α–Ketoisocaproate

To determine the ^{13}C enrichment of plasma α–ketoisocaproate, *o*-trimethylsilyl quinoxalinol derivatives were prepared using *bis*-(trimethylsilyl)trifluoroacetamide (Regis Chemical Company or Sigma Chemical Company Ltd, Poole. U.K.) (Table 3.5) (Rocchiccioli *et al*, 1981). Gas chromatography-mass spectrometry was by analogous methods to these used for

plasma amino acid analysis with monitoring of ion-currents m/z 232 and 233. Ketovalerate was included as internal standard to quantify the α-ketoisocaproate.

3.12 Isotope Ratio Mass Spectrometry

The enrichment of ^{13}C in carbon dioxide was measured with an automated Finnigan MAT breath gas analysis system attached to a Finnigan Delta D isotope ratio-mass spectrometer.

3.12.1 ^{13}C analysis of Carbon Dioxide in Breath and Blood

Carbon dioxide in breath was cryogenically purified on-line before measurement of enrichment (Scrimgeour and Rennie, 1988). Analysis of ^{13}C enrichment of carbon dioxide in whole blood was performed with the same equipment after liberation of the carbon dioxide with orthophosphoric acid. The total blood concentration of carbon dioxide was measured at the same time by comparing the pressure of the carbon dioxide released from the blood into the evacuated sample tubes with that from a range of carbonate standards prepared in the same way (Scrimgeour and Rennie, 1988).

3.12.2 ^{13}C Analysis of Protein-Bound Leucine

To prepare the muscle samples for the determination of [1-^{13}C]leucine enrichment, the samples were freeze-dried, ground and free amino acids were extracted with ice-cold perchloric acid and the residue hydrolysed in 6 mol/l hydrochloric acid (Tables 3.6.1 and 3.6.2). Leucine was separated by preparative gas chromatography (Smith *et al*, 1988). The carboxyl carbon of the isolated leucine

Table 3.3. Determination of Isotope Ratio of Plasma Leucine and Phenylalanine

This analysis was based on the method of Mawhinney *et al* (1986) and employed a *t*-butyldimethylsilyl derivative, separation by gas chromatography and isotope quantification by mass spectrometry in the electron impact mode.

Method

1) To a microcentrifuge tube add 600 µl of plasma and 20 µl of urease solution (Urease from jack bean, Type IX, 500 units/ml in 0.01 mol/l phosphate buffer, pH 6.7 with sodium azide 1 µmol/l), vortex to mix and incubate at 37°C for 15 min. For samples where high leucine and phenylalanine concentrations are anticipated the initial volume should be appropriately reduced.
2) To precipitate the proteins, add 700 µl methanol/HCl (4:1 v/v), vortex to mix and centrifuge at high speed for 5 min.
3) Decant supernatant to a screw-top microcentrifuge tube and remove the solvent by overnight rotary-evaporation.
4) Add 200 µl pyridine and 50 µl *N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide, cap tightly and vortex.
5) Incubate at 80°C for 60 min total; remove from the oven after 30 min, vortex to mix and return to the oven.
6) Add 400 µl decane, vortex and centrifuge for 3 min at high speed. Transfer to autosampler vials.

was liberated as carbon dioxide by ninhydrin and its ^{13}C enrichment was determined with the breath gas system, optimised for small quantities of carbon dioxide (Scrimgeour *et al,* 1988a).

3.13 Amino Acid Concentrations in Plasma and Muscle

Amino acid concentrations were determined in sulphosalicylic acid extracts of plasma and perchloric acid (0.5 mol/l) extracts of muscle. Automated amino acid analyzers were used employing either a) ion-exchange chromatography with elution and separation of amino acids with strongly acidic lithium citrate buffers, norvaline as internal standard, post-column derivatization with *o*-pthalaldehyde/-2-mercaptoethanol and fluorometric detection (LC5000, Biotronic GmbH, Munchen, F.D.R.); b) ion-exchange chromatography, lithium citrate buffers, no internal standard, post-column derivatization with ninhydrin and photometric detection (LC5000); or c) reversed-phase high-pressure liquid chromatography (HPLC), norvaline as internal standard, pre-column on-line derivatization with *o*-pthalaldehyde/2-mercaptoethanol and fluorometric detection (LKB Bromma, Sweden) (Graser *et al*, 1985). The HPLC system column (Spherisorb ODS II, 3 µm, 125 mm by 4.6 mm) was equipped with a guard column (10 mm by 4.6 mm) filled with the same material. Elution was performed with a sodium phosphate buffer (12.5 mmol/l, pH 7.2)- acetonitrile gradient. All the samples from every patient within a study series were analyzed with the same amino acid analyzer. Calculation of the values for the intramuscular concentration was done on the assumption that 13% of the water content, by weight, was intracellular.

3.14 Plasma Glucose Analysis

Concentration of plasma glucose was determined with a Glucose Analyzer 2 (Beckman Instruments, Irvine, CA, U.S.A.), which employed a glucose oxidase reaction. With this instrument plasma glucose could be analyzed within 80 sec of

blood sampling, allowing rapid adjustments to be made to the glucose infusion rate during the euglycaemic insulin clamp studies.

3.15 Plasma Hormone Concentrations

Plasma hormones were measured by radioimmunoassay. Insulin was determined with a double- antibody kit (Ire-Medgenix Sa, Brussels, Belgium); the primary incubation period was increased to 20 h to optimise the sensitivity of the assay. Plasma free insulin concentration, in extracts prepared at the bedside, was measured with the same radioimmunoassay procedure, but the kit standards were treated with polyethylene glycol- phosphate buffer solution in an identical manner to the samples from the insulin treated patients. Glucagon was measured with a double-antibody kit (ICN Biomedicals Inc, Carson, CA, U.S.A.), cortisol was measured with an antibody- coated-tube kit (Immunodiagnostics Systems Ltd, Washington, U.K.) and insulin-like growth factor 1 with a double- antibody kit (Nicols Institute Diagnostics, San Juan Capistrano, CA, U.S.A.). C-peptide, measured at the start of the diabetic studies only, was with a double-antibody kit (Ire-Medgenix Sa).

3.16 D-(-)-3-Hydroxybutyrate and Lactate Analysis

The blood concentration of D-(-)-3-hydroxybutyrate was measured by an nicotinamide-adenine dinucleotide-linked 3-hydroxybutyrate dehydrogenase enzyme assay (Williamson and Mellanby, 1974) and lactate was by an analogous technique with lactate dehydrogenase (Gutmann and Wahlefeld, 1974).

3.17 Blood Gas Analysis

The PO_2, oxygen saturation, pH and total bicarbonate concentrations of arterial and venous blood was measured using a Corning 189 blood gas analyzer (Corning Medical, Medfield, Massechusits, U.S.A.).

3.18 Statistical Analysis

In the text, tables and figures mean values and standard errors are presented. In some studies for D-(−)-3-hydroxybutyrate and hormones the results were transformed to their natural logarithms before statistical analysis: The means presented are the $\log_e$ transformations of the logarithmic means; the ranges are the natural of values of the 95% confidence limits below and above the $\log_e$ means. Results from the two phases within studies were compared with the two-tailed Student's *t*-test for paired data. A *t*-test for means was used to compare different study groups. A stepwise regression analysis was performed to compare rates of protein synthesis with indices of insulin sensitivity and substrate supply. A microcomputer-based statistics package was used for the calculations (Microstat, Ecosoft Inc, Indianapolis, IN, U.S.A.).

Table 3.4. Determination of Isotope Ratio of Intramuscular Free Leucine

This analysis was also based on the method of Mawhinney *et al* (1986) and employed a *t*-butyldimethylsilyl derivative, separation by gas chromatography and isotope quantification by mass spectrometry in the electron impact mode.

Method

1) A weighed sample of muscle (about 100-300 mg) is lyophilized overnight and then ground with a glass rod in a plastic test tube.
2) Extract free amino acids by adding 3 ml perchloric acid (0.5 mol/l, with norvaline 64 µmol/l to quantify amino acids using the amino acid analyzer), vortex and stand in ice for 30 min.
3) Centrifuge for 20 min (3000 RPM, 4°C) and decant supernatant. The isotope ratio of protein-bound leucine is determined in the residue (Table 3-6).
4) To 500 µl of the supernatant add one drop of universal pH indicator and neutralise with potassium bicarbonate (2 mol/l). Vortex, let stand in ice for 20 min and vortex again.
5) Centrifuge (3000 RPM, 20 min, 4°C) and pipette off supernatant. Mix with an equal volume of glacial acetic acid. Apply to a 2.5 ml bed of ion-exchange resin (Dowex 50W-X8(H^+), 100-200 mesh), prewashed in HCl (1 mol/l).
6) The column is washed with HCl (2x1 ml) and then distilled deionized water (2x1 ml).
7) The amino acids are eluted with 3 ml of ammonia solution (4 mol/l) and are collected in a screw-top microcentrifuge test tube.
8) Remove the ammonia by overnight rotary-evaporation.
9) Add 50 µl pyridine and 50 µl *N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide, cap tightly and vortex.
10) Incubate at 80°C for 60 min total; remove from the oven after 30 min, vortex to mix and return to the oven.
11) Add 50 µl decane, vortex and centrifuge for 3 min at high speed. Transfer to autosampler vials.

Table 3.5. Determination of Isotope Ratio of Plasma α-Ketoisocaproate

This analysis used the method of Rocchiccioli *et al* (1981), further modified by Ford *et al* (1985), employing a quinoxalinol derivative, separation by gas chromatography and isotope quantification by mass spectrometry in the electron impact mode.

Method

1) To a micro-centrifuge tube add 600 µl plasma, 30 µl ketovaleric acid solution (0.1 mg per ml) and 800 µl ethanol to precipitate the protein. Vortex to mix.
2) Centrifuge at high speed for 3 min, decant the supernatant to a test tube and dry under a stream of nitrogen at 50°C.
3) To the residue add 200 µl distilled water and 100 µl *o*-phenylenediamine solution (2% w/v in HCl 4 mol/l).
4) Heat in the oven for 60 min at 90°C, then cool.
5) Extract with 2 by 1 ml aliquots of ethylacetate and dry the combined extracts over anhydrous sodium sulphate.
6) Decant the dried ethylacetate to a reaction vial, taking care not to transfer any sodium sulphate. Dry under nitrogen at room temperature.
7) Dissolve the residue in 100 µl pyridine. Add 100 µl *bis*-(trimethylsilyl)-trifluoroacetamide, cap tightly and heat at 120°C for 30 min.
8) Cool vials and evaporate to dryness under nitrogen at room temperature.
9) Dissolve the residue in 500 µl decane containing 5% *bis*-(trimethylsilyl)-trifluoroacetamide and transfer to an autosampler vial.

Table 3.6.1. Determination of Isotope Ratio of Muscular Bound Leucine

This analysis was based on the method of Smith *et al* (1988) where ground muscle protein is washed and acid-hydrolysed, leucine is separated by preparative gas chromatography of trifluoroacetyl isobutyl esters, and isotope quantification is by isotope ratio mass spectrometry of the carboxyl carbon after liberation as CO_2 with ninhydrin.

Method

Protein Extraction

1) A weighed sample of muscle (about 100-300 mg) is lyophilized overnight and then ground with a glass rod in a plastic test tube.
2) Extract free amino acids by adding 3 ml perchloric acid (0.5 mol/l), vortex and stand in ice for 30 min. Centrifuge for 20 min (3000 RPM, 4°C) and decant supernatant.
3) To the residue add a further 3 ml perchloric acid (0.2 mol/l), vortex to mix and decant the supernatant. Repeat two times.
4) Add 3 ml NaOH (0.5 ml), mix and incubate at 37°C for 60 min in a water bath. Remove 100 µl, which can be used for a protein assay. Add 2 perchloric acid (1 mol/l) and centrifuge (3000 RPM, 20 min).
5) Suspend in 2 ml of perchloric acid (0.2 mol/l), vortex, centrifuge and decant supernatant.
6) Add 5 ml perchloric acid (1 mol/l) resuspend the pellet and heat at 70°C for 30 min in a water bath. Centrifuge (3000 RPM, 20 min) and decant supernatant.
7) Suspend the protein pellet in 3 ml HCl (6 mol/l) and transfer to a hydrolysis tube. Heat at 120°C overnight.

Ion-Exchange Chromatography

8) Transfer to a centrifuge tube and remove the solvent by rotary evaporation. Dissolve the residue in 50% glacial acetic acid.
9) Apply to a 2.5 ml column of ion-exchange resin (Dowex 50W-X8(H^+), 100-200 mesh, prewashed with 1 mol/l HCl). Rinse out the hydrolysis tube with 2x1 ml HCl (1 mol/l). Rinse the column with deionized distilled water (2x1 ml).
10) Elute the amino acids with 3 ml ammonia solution (4 mol/l). Collect the eluent into reaction vials, and blow-off the ammonia under nitrogen for about 30 min at room temperature (until pH is 7-9). Freeze with liquid nitrogen and lyophilize overnight.

Table 3.6.2. Determination of Isotope Ratio of Muscular Bound Leucine

Derivatization Procedure

11) Add an aliquot of dichloromethane (500 µl) and dry by evaporation under nitrogen at room temperature. Dissolve the residue in 400 µl 3 mol/l HCl-isobutanol and heat at 110°C for 30 min. Allow to cool and evaporate under nitrogen at room temperature.
12) Dissolve the residue in ethyl acetate (250 µl) and trifluoroacetic anhydride (100 µl) and react at 120-130 min for 30 min, releasing the pressure after 15 min. Allow to cool and evaporate off solvent under nitrogen at room temperature.
13) Dissolve in 100 µl of n-heptane and transfer to an autosampler vial. Reduce the volume to 100 µl under nitrogen at room temperature.

Preparative Gas Chromatography

14) An aliquot (50-100 µl) of sample is injected into the injection port of a Pye 304 chromatograph fitted with a wide-bore glass column (6 mm inside diameter x 4.6 m) packed with 2% EGA on 60-80 mesh Chromosorb W AW (Phase Sep, Queensferry, Clwyd, U.K.) and with a post column splitter (split ratio 99:1).
15) Argon was the carrier gas at a flow of 30 ml/min. The temperature of the injector was 200°C and the oven was programmed from 150 to 165°C at 2 °C/min, then held at 165°C for 6 min before being increase to 220°C to drive off later eluting amino acid derivatives.
16) The leucine derivative is condensed in a demountable U-trap, cooled with liquid nitrogen. The leucine fraction is identified by comparison of retention times with these of leucine standards (about 11.5 min).
17) The leucine derivative is hydrolysed by adding 100 µl KOH (1 mol/l) and heating at 90°C for 45 min or leaving at room temperature overnight. The sample is transferred 2 ml of buffer (pH 2) to a Vacutainer and is stored at 4°C overnight.
18) The vacutainer is cooled in ice before adding ninhydrin (25 mg) and a drop of antifoaming agent. After degassing and sealing the sample is heated in a water bath at 90°C for 30 min to liberate the carboxyl-C as CO_2 for isotope ratio mass spectral analysis (Scrimgeour *et al*, 1988a).

Chapter 4. INCREASE IN ANTERIOR TIBIALIS MUSCLE PROTEIN SYNTHESIS IN HEALTHY MAN DURING MIXED AMINO ACID INFUSION: INCORPORATION OF [1-^{13}C]LEUCINE

CONTENTS

TABLES

FIGURES

4.1 Summary

Anterior tibial muscle protein synthesis in seven healthy postabsorptive men was determined from increases in muscle protein bound leucine enrichment during a primed continuous infusion of L-[1-^{13}C]leucine. Biopsies were taken 30 min after the beginning of leucine infusion (when plasma ^{13}C enrichment was steady), 240 min later during continued fasting and again after 240 min of infusion of a mixed amino acid solution which increased plasma total amino acid concentrations by 37%. The mean enrichment of ^{13}C in plasma α-ketoisocaproate was used as an index of the enrichment of the precursor pool for leucine metabolism. Anterior tibial muscle mixed protein synthetic rate during fasting was 0.055±0.003 (Mean±SEM) %/h and this increased by an average of 35% during infusion of mixed amino acid to 0.074±0.008 %/h (P<0.05). Whole-body protein breakdown (expressed as the rate of endogenous leucine appearance in plasma) was 121±3 μmol•kg^{-1}•h^{-1} during fasting and decreased (P<0.01) by an average of 12% during amino acid infusion. Leucine oxidation was 18±1 μmol•kg^{-1}•h^{-1} during fasting and increased (P<0.001) by 89% during amino acid infusion. Whole-body protein synthesis (non-oxidative leucine disappearance) was 104±2 μmol•kg^{-1}•h^{-1} during fasting and rose by 13% (P<0.001) during mixed amino acid infusion.

Enrichment of muscle free leucine was only 61±7% of that in plasma α-ketoisocaproate and this increased to 74±7% (P<0.02) during mixed amino acid infusion.

The results suggest that increased availability of amino acid reverses whole-body protein balance from negative to positive and a major component of this is the increase in muscle protein synthesis.

4.2 Introduction

Studies of quadriceps muscle protein synthetic rates in man have shown increases during mixed oral feeding (Rennie *et al*, 1982b; Halliday *et al*, 1988). In studies of amino acid exchange across human limbs, an apparent increase in protein synthesis but no change in protein breakdown occurred during mixed oral feeding which should elevate insulin secretion (Cheng *et al*, 1985; Cheng *et al*, 1987). In contrast, insulin infusion in fasting healthy subjects not only did not induce an increase in indirect indices of protein synthesis but reduced protein breakdown (Gelfand and Barrett, 1987). Infusion of insulin in diabetic patients did not increase quadriceps muscle protein synthesis but did markedly reduce plasma amino acid concentrations (Pacy *et al*, 1989). These observations suggest that amino acids *per se* may regulate muscle protein synthesis.

The individual contributions of amino acids, energy (i.e. equivalents of carbohydrate and lipids) and other paracrine and endocrine hormones in the control of skeletal muscle protein synthesis in man are not known. It is known that in man, infusion of substantial amounts of either leucine (Schwenk and Haymond, 1987) or mixed amino acids (Castellino *et al*, 1987; Tessari *et al*, 1987) increased whole-body protein synthesis but only a mixed amino acid infusion reduced whole-body breakdown, and although the branched chain amino acids and leucine in particular are said to have anabolic effects on animal muscle (May and Buse, 1989) there was no data for human muscle *in vivo*.

From the whole-body protein turnover results in man and the results from *in vitro* studies we formulated the hypothesis that amino acids would increase skeletal muscle protein synthesis in man. If this was the case it would in part

explain the apparent inability of insulin to increase skeletal muscle protein synthesis in man (Pacy *et al*, 1989) as decreased amino acid availability occurs during insulin infusion. The aim of the study reported here was to study the effects of an intravenous infusion of mixed amino acids without additional fuel substrates or hormones on the skeletal muscle protein synthetic rate in postabsorptive healthy man. A subsidiary aim was to whole-body protein turnover and to examine the changes induced by provision of amino acids.

4.3 Methods

4.3.1 Subjects

Seven healthy men (age range 24.5-39.5, mean 29.5±1.9 years; weight 73.1±1.2 kg; body mass index 23.8±0.3 kg/m^2) were studied.

4.3.2 Protocol

A muscle biopsy based protocol was employed (Protocol a, Section 3.10.1, Figure 3.1). After an overnight (15 h) fast a dorsal hand vein was cannulated for blood sampling and a contralateral forearm vein was cannulated for all infusions. Basal blood and breath samples were obtained (time -15 min). A primed (7.17±0.19 μmol/kg) continuous (7.39±0.09 μmol•kg^{-1}•h^{-1}) infusion of L–[1–^{13}C]leucine was established and $NaH^{13}CO_3$ (1.86±0.03 μmol/kg) injected intravenously. Thirty min later (time 30 min) an anterior tibialis muscle biopsy was obtained (Section 3.7) and a second biopsy was obtained after a further 240 min (time 270 min). A third muscle biopsy was obtained after a further 240 min period (510 min). After the second muscle biopsy a priming dose of a commercial preparation of mixed amino

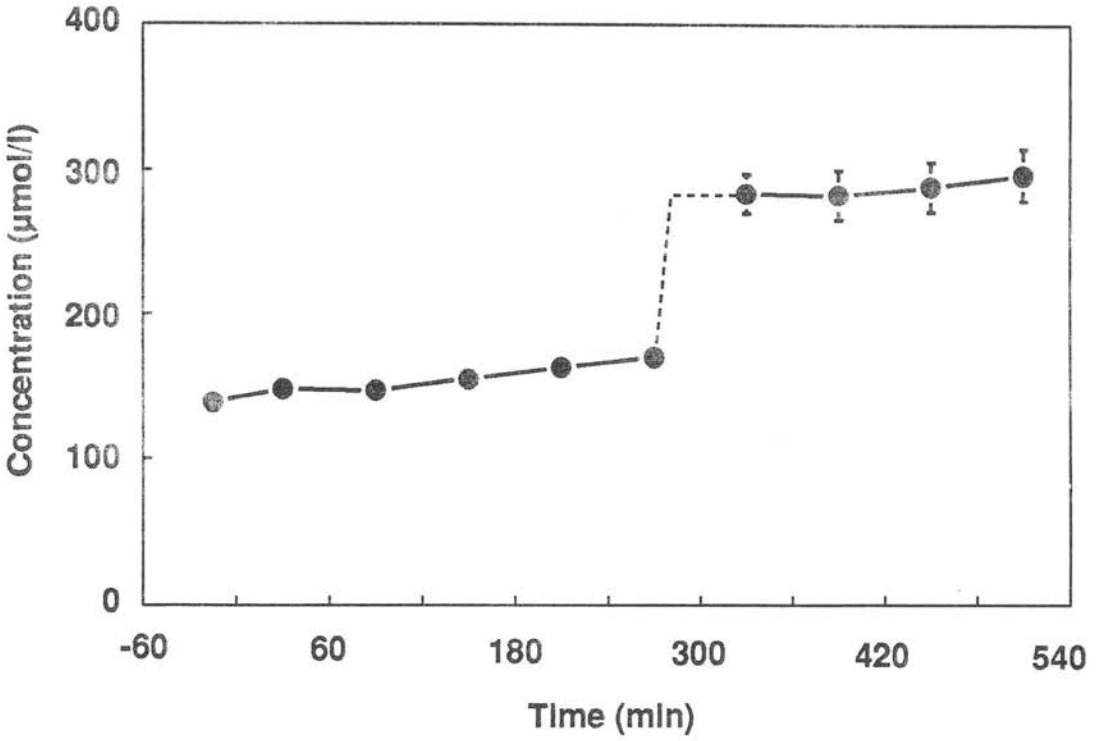

Figure 4.1. Plasma leucine concentration in the absence (-15 to 270 min) and in the presence (270 to 510 min) of a mixed amino acid infusion

acids (Synthamin 9, Section 3.3, Table 3.1) was given (83.5 mg of amino acids/kg over 10 min), with a second bolus of $NaH^{13}CO_3$ (0.94±0.01 µmol/kg); amino acids were continuously infused until the end of the study at a rate of 1.52 $ml \cdot kg^{-1} \cdot h^{-1}$ providing 83.5 mg of amino $acids \cdot kg^{-1} \cdot h^{-1}$, 46.5 µmol of $leucine \cdot kg^{-1} \cdot h^{-1}$ and 0.33 g of $nitrogen \cdot kg^{-1} \cdot day^{-1}$. Arterialized venous blood samples, with the hot-box technique (Section 3.5), and breath samples (Section 3.6) were obtained each hour. Indirect calorimetry was undertaken for 11 min each hour (Section 3.6). Amino acid analysis was by automated ion exchange chromatography with fluorometric detection (Section 3.13).

4.4 Results

4.4.1 Indirect Calorimetry

Resting energy expenditure was 5.42±0.28 kJ/min in the absence of the amino acid infusion and increased to 5.87±0.27 kJ/min ($P<0.01$) during the infusion. No change was found in the respiratory quotient (0.73 overall).

4.4.2 Substrates and Hormones

Values of the concentrations of plasma glucose, insulin, glucagon and cortisol and of blood D-(-)-3-hydroxybutyrate were stable within each study period, without and with amino acid infusion (Table 4.1). During amino acid infusion insulin was 53% greater ($P<0.05$) and glucagon 41% greater ($P<0.01$) compared with postabsorptive concentrations. Concentrations of glucose, D-(-)-3-hydroxybutyrate and cortisol were not affected by the amino acid infusion.

4.4.3 Amino Acid Concentrations

The mean plasma leucine concentration (Figure 4.1, Table 4.2) in the absence of amino acid infusion, from 30 to 270 min, was 157±8 μmol/l; a rise ($P<0.05$) of 16% occurred from 90 to 270 min. During amino acid infusion leucine increased by nearly 80% to a plateau value of 278±17 μmol/l ($P<0.001$) between 330 and 510 min. During fasting the concentration of free leucine in intramuscular fluid (167±8 μmol/l was not different from that in plasma: this increased by 52% to 254±21 μmol/l ($P<0.02$) during amino acid infusion when there was no statistical difference between plasma and intramuscular free leucine concentrations. Total

Table 4.1. Substrate and Hormone Concentrations in Blood and Plasma in the Absence and in the Presence of Amino Acid Infusion

	Postabsorptive	Amino acid infusion
Blood D-(-)-3-hydroxybutyrate (mmol/l)	0.14±0.03	0.16±0.02
Plasma glucose (mmol/l)	4.7±0.1	4.9±0.1
Plasma insulin (pmol/l)	44±11	68±12[a]
Plasma glucagon (pmol/l)	36±6	50±6[b]
Plasma cortisol (nmol/l)	279±37	249±77

Results are means±SEM over time. Statistical significance: [a]$P<0.05$, [b]$P<0.01$.

plasma amino acid concentration was 2890±100 μmol/l initially and a rise ($P<0.001$) of 37% to 3950±140 μmol/l occurred during amino acid infusion. During infusion no change was found in the plasma concentrations of aspartic acid and glutamine; reductions occurred in glutamic acid and taurine, amino acids not present in the mixture infused.

4.4.4 Plasma and Muscle Free Leucine Pool Enrichments

For both plasma leucine and α-ketoisocaproate, plateau enrichment was achieved during the last 180 min of each phase of the study (Figure 4.2, Table

Table 4.2. Amino Acid Concentrations in Plasma and Intramuscular Water (IMW)

	Amino acid concentration (μmol/l)			
	Plasma (30-270)	IMW (270)	Plasma (300-510)	IMW (510)
Alanine	253±17	1610±200[c]	467±25[c]	1900±110[c]
Arginine	101±9	341±35[c]	211±17[c]	473±50[c]
Aspartic acid	8±2	833±130[c]	8±2	926±92[c]
Glutamic acid	82±14	5210±200[c]	70±12[d]	5580±350[c]
Glutamine	673±17	11700±900[c]	658±8	12100±700[c]
Glycine	237±12	1450±180[c]	405±16[c]	1600±110[c]
Histidine	92±4	369±20[c]	138±8[c]	412±17[c]
Isoleucine	55±3	59±6	160±6[c]	145±10
Leucine	157±8	167±12	278±17[c]	254±21
Lysine	181±19	616±45[c]	241±20[c]	679±63[c]
Methionine	19±1	--	63±2[c]	--
Phenylalanine	54±4	85±9[b]	103±7[c]	132±8[a]
Serine	124±5	833±130[c]	186±5[c]	926±92[c]
Taurine	71±5	20400±1100[c]	60±3[e]	19800±1200[c]
Threonine	128±7	573±66[c]	181±7[c]	641±38[c]
Tryptophan	44±3	4870±1060[c]	65±5[c]	7310±890[c]
Tyrosine	47±3	71±1[a]	48±2	53±5
Valine	254±18	511±54[b]	380±23[c]	664±55[b]
Total amino acids	2890±100	52400±3000[c]	3950±140[c]	55900±1600[c]

Values for plasma and IMW at the same time points are compared, and also values for plasma at different times (increases: [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$; reductions: [d]$P<0.02$, [e]$P<0.01$).

4.3). The ratio of ^{13}C enrichment in plasma α-ketoisocaproate relative to that in plasma leucine was 83±2% in the absence of exogenous amino acids and this was maintained (85±3%) during amino acid infusion. The ^{13}C enrichment in muscle free leucine was 4.26±0.59 APE 30 min after the [^{13}C]leucine bolus (time 30 min) and was 3.45±0.42 APE at the end of the first phase of the study before infusion of the amino acids. This enrichment was unchanged (3.41±0.81 APE) during amino acid infusion despite reductions in the enrichment of ^{13}C in both plasma leucine and plasma α-ketoisocaproate (Table 4.3). The ^{13}C enrichment of intramuscular free leucine was 51±3% of that of plasma leucine in the absence of exogenous amino acids and rose to 63±5% ($P<0.02$) during amino acid infusion. The ^{13}C enrichment of intramuscular free leucine was 61±7% of that of α-ketoisocaproate in the absence of exogenous amino acids and rose to 74±6% ($P<0.02$) during their infusion.

4.4.5 Muscle Protein Synthesis

The rate of anterior tibial muscle protein synthesis (Table 4.4, Figure 4.3) in the absence of amino acid infusion, calculated on the basis that the mean ^{13}C enrichment in plasma α-ketoisocaproate (Table 4.3) should closely represent the precursor synthetic pool and provide a best estimate of protein synthesis, was 0.055±0.003 %/h; the rate increased during amino acid infusion by 35% to 0.074±0.008 %/h ($P<0.05$). These values should be considered as the best estimate of protein synthesis. When the synthetic rate was calculated on the basis of plasma leucine enrichment as representative of the precursor pool enrichment, this minimal estimate of muscle protein synthesis was 0.045±0.003 %/h in the absence of amino acid infusion, 18% slower ($P<0.001$) than the plasma α-ketoisocaproate based

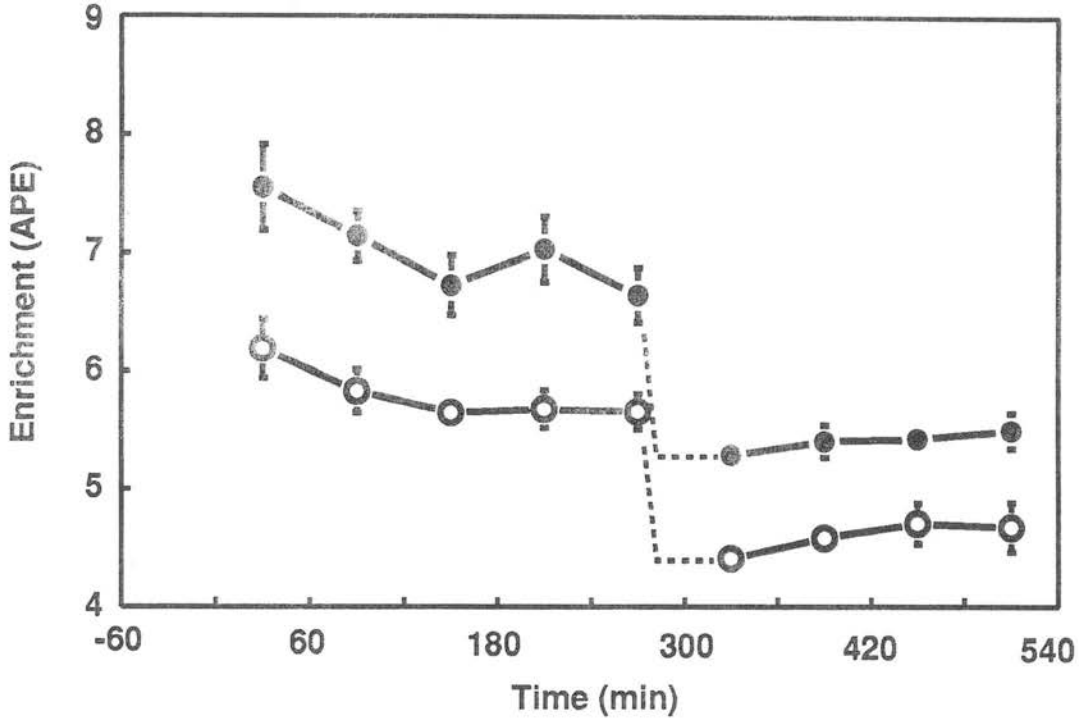

Figure 4.2. Plasma leucine (solid circles) and α-ketoisocaproate (hollow circles) enrichments in the absence and in the presence of mixed amino acid infusion

values; a rise of 40% to 0.063±0.007 %/h ($P<0.05$) occurred during amino acid infusion. In contrast to the α–ketoisocaproate based rates these plasma leucine-based values should be considered as minimal estimates of protein synthesis. When calculated on the basis of the ^{13}C enrichment in intramuscular free leucine, taken as representative of the precursor pool enrichment, this maximal or overestimate of muscle protein synthesis was 0.094±0.017 %/h in the absence of amino acid infusion (71% faster (NS) than the plasma α–ketoisocaproate based rate). During amino acid infusion, the rate of muscle protein synthesis calculated in this fashion

(0.107±0.017 %/h) showed no significant difference from that calculated in the absence of exogenous amino acids.

4.4.6 Whole-Body Leucine Kinetics

During the infusion a rise (P<0.001) of 26% occurred in the plasma leucine flux (Table 4.5), but this increase was less than the exogenous leucine infusion rate because of a reduction (P<0.01) of 12% in the rate of appearance of endogenous leucine, a measure of protein breakdown. Whole-body leucine oxidation increased (P<0.001), in absolute terms, to almost double the postabsorptive value during amino acid infusion; as a proportion of plasma leucine flux oxidation rose from 15% to 22%. The non-oxidative rate of leucine disappearance, i.e. whole-body protein synthesis, increased (P<0.001) by 13% during the amino acid infusion. Net leucine uptake changed (P<0.001) from negative to positive, suggesting that net protein balance had become positive.

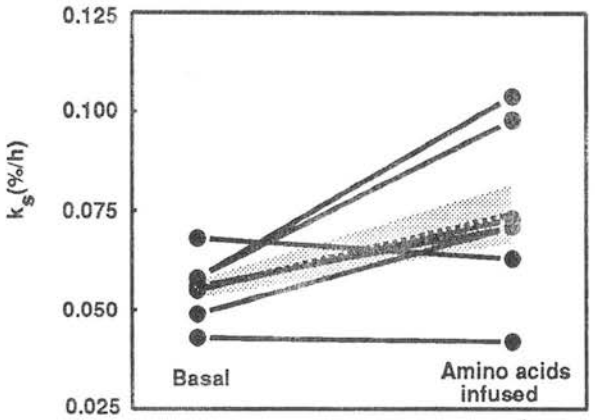

Figure 4.3. Skeletal muscle fractional protein synthesis in the absence and in the presence of mixed amino acid infusion calculated from α-ketoisocaproate

4.5 Discussion

This study shows, for the first time, the effects of parenteral infusion of mixed amino acids on anterior tibial muscle protein synthesis. Information is also provided about the relationships of leucine and α–ketoisocaproate in the plasma pool and in muscle intracellular water of anterior tibialis.

In essence the results show that increases in amino acid availability in plasma and muscle stimulate muscle protein synthesis, calculated on the basis of the enrichments of plasma α–ketoisocaproate or leucine, but not when calculated on the basis of intracellular leucine enrichment. The correct interpretation of these results depends on knowledge of the relative tracer labelling of muscle leucine-tRNA, plasma leucine, intramuscular free leucine, and the keto acid of leucine, information not available for man. In previous studies of human muscle protein synthesis (Rennie *et al*, 1982b; Gibson *et al*, 1987; Halliday *et al*, 1988; Nair *et al*, 1988a; Nair *et al*, 1988b; Pacy *et al*, 1989) the plasma α–ketoisocaproate enrichment has generally been employed as the basis of calculations, since a number of studies of precursor pool labelling of leucine in skeletal and cardiac muscle, both *in vitro* and *in vivo*, showed tRNA labelling to be nearer to that of the extracellular, than to that of the intracellular, amino acid pool (Section 2.3.1). In contrast one other study reports the opposite result (Schneible *et al*, 1981). Studies using flooding doses of leucine in the rat *in vitro* suggest that the precursor pool for protein synthesis lies between that of plasma leucine and intracellular free leucine (Obled *et al*, 1989). The substantial transamination of leucine in human forearm arteriovenous exchange studies (Cheng *et al*, 1985;

Cheng *et al*, 1987) provides further evidence of rapid entry of extracellular leucine to intracellular metabolic processes.

Table 4.3. ^{13}C Enrichment of Free Leucine and its Metabolites in Plasma, Muscle and Breath, and the rate of Increase in Muscle Protein-Bound Leucine

	Postabsorptive (30-270 min)	Amino acid infusion (330-510 min)
Plasma leucine (APE)	7.03±0.21	5.41±0.08[a]
Plasma α-ketoisocaproate (APE)	5.80±0.12	4.60±0.14[a]
Muscle free leucine (APE)[b]	3.45±0.42	3.41±0.31
Expired CO_2 (APE)	0.0129±0.0007	0.0185±0.0009[a]
10^3•Protein bound leucine/time[c] (APE/h)	3.20±0.19	3.41±0.35

Statistical significance: [a]$P<0.001$. [b]Muscle free leucine results were obtained at either 270 or 510 min of elapsed time. [c]The rate of increase of protein-bound leucine enrichment was calculated from changes calculated over 30-270 min and 270-510 min.

The relative increase in the equilibrium between the intramuscular free leucine enrichment and that of plasma leucine during amino acid infusion is partly a function of the reduced arteriovenous decrement in leucine enrichment that occurs when plasma leucine concentration is increased (Cheng *et al*, 1985; Cheng

et al, 1987; Layman and Wolfe, 1987) and partly to the increased flux of leucine (Cheng *et al*, 1985; Cheng *et al*, 1987) in the fed state. The initial (time 30 min) higher intracellular leucine enrichment may have been a transient effect due to the bolus of [^{13}C]leucine or to an increase in protein breakdown during the initial period of the study. It remains a possibility that the lack of a significant effect of amino acid infusion on muscle protein synthesis, calculated on the basis of the free muscle leucine labelling, was a type 2 error: this may have occurred as a result of taking an insufficiently long period over which to measure the enrichment of muscle protein, or to the effect of the relative methodological precision of measurements made on small samples of free and protein bound intramuscular leucine with a resulting greater variability of the calculated protein synthetic rates. Until information is available of the leucyl-tRNA charging of human skeletal muscle, rates of muscle protein synthesis derived from plasma α–ketoisocaproate enrichment should be viewed as the best available estimates, rates derived from plasma leucine enrichment as minimal estimates and rates derived from intracellular leucine enrichment as overestimates.

Complete mixtures of amino acids are reported to enhance protein synthesis in cell free systems suggesting that there is a direct stimulatory effect of amino acids on protein synthesis (Tyobeka and Manchester, 1985). There is, however, no known mechanism for this effect and the K_m for the charging of tRNA is likely to be much lower than the intramuscular concentration of amino acids so that tRNA is always fully saturated (Tyobeka and Manchester, 1985). Leucine has been reported to stimulate rat muscle protein synthesis *in vitro* (Buse and Reid, 1975; May and Buse, 1989) and whole body protein synthesis in man (Schwenk and

Haymond, 1987). There is an effect of the branched chain amino acids to control initiation of protein synthesis by increasing the proportion of muscle ribosomes as polyribosomes (Buse *et al*, 1979; Flaim *et al*, 1982b; May and Buse, 1989). However, branched-chain amino acids have no effect on muscle amino acid balance (Hagenfeldt *et al*, 1980; Abumrad *et al*, 1982; Eriksson *et al*, 1983; Morrison *et al*; 1988c) or ribosome profile in man (Hammarqvist *et al*, 1988). Our results showed that amino acid infusion caused an increase in intramuscular leucine and other branched chain amino acid concentrations so that if they were anabolic there was scope for the effect to be expressed.

To what extent are the increases in muscle protein synthesis likely to result from the small increases in insulin? Many studies in animals both *in vitro* and *in vivo* (Section 1.8.1) provide evidence of a stimulatory effect of insulin on muscle protein synthesis, most sensitive in the range 0-15 mU/l (Millward *et al*, 1983). Studies in adult man conflict with these animal-based results suggesting that insulin may not have an anabolic effect (Gelfand and Barrett, 1987; Pacy *et al*, 1989). An increase occurred in glucagon but this hormone certainly does not stimulate muscle protein synthesis at the concentrations found and may decrease it at pathophysiological concentrations (Preedy and Garlick, 1988).

It remains possible that growth hormone itself induced the reported changes as this hormone is shown to have an acute, direct insulin-like effect on rat muscle protein synthesis *in vitro* (Cameron *et al*, 1988). Insulin-like growth factors, expressed by messenger ribonucleic acids in most human foetal tissues including muscle (Han *et al*, 1988), may have exerted a paracrine influence to modulate increases in protein synthesis in response to amino acid provision, but during

Table 4.4. Calculated Values of Anterior Tibial Muscle Protein Synthetic Rate According to Choice of Leucine Pool to Represent the Precursor Pool for Protein Synthesis

Precursor pool assigned	Postabsorptive	Amino acid infusion	*P* value
Plasma α-ketoisocaproate (%/h)	0.055±0.003	0.074±0.008	0.05
Plasma leucine (%/h)	0.046±0.003	0.063±0.007	0.05
Intramuscular leucine (%/h)	0.094±0.017	0.107±0.017	NS

NS, non significant.

infusion of the same amount of amino acids in another study we were unable to measure any change in plasma insulin-like growth factor 1 concentration (Chapter 5). Since, the anabolic effect of the exogenous amino acids on muscle cannot be firmly identified with changes in availability of insulin, glucagon (or cortisol, glucose and ketone bodies, none of which changed in concentration), we are therefore forced to the conclusion that either the simple increase in intramuscular concentration of amino acids or an increase in some other unknown anabolic factor caused the observed increase in muscle protein synthesis. The present results strongly suggest that a major component in the two fold increase seen in muscle protein synthesis in going from the postabsorptive to the fed state (Rennie *et al*,

1982b; Halliday *et al*, 1988) is simply due to an increased availability of amino acids.

Table 4.5. Whole-Body Leucine Kinetics

	Postabsorptive ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	Amino acid infusion ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	Change (%)
Infusion rate	--	46.5	--
Flux	121±3	153±5[b]	+26
Protein breakdown	121±3	106±5[a]	-12
Oxidation	13±1	34±2[b]	+88
Protein synthesis	104±2	118±3[b]	+13
Net protein balance	-18±1	+12±2[b]	

Statistical significance: [a]$P<0.01$, [b]$P<0.001$.

The values in the postabsorptive state for anterior tibialis mixed protein synthesis are somewhat higher than those for quadriceps (0.046±0.003 %/h) obtained with identical methods and methods of calculation, by ourselves and other workers (Gibson *et al*, 1987; Halliday *et al*, 1988). This difference may be due to the greater type 1 fibre content of anterior tibial muscle (73±4%) compared with quadriceps (47±4%) (Johnson *et al*, 1973). At present the relative synthetic rates for human type 1 and type 2 muscle fibres are not known but it is known that in

animals muscles with predominantly type 1 fibre composition turn over faster than those predominantly type 2 (Goldberg, 1967; Flaim *et al*, 1980; Preedy and Garlick, 1988).

The absolute values, and the changes that occurred between the postabsorptive and amino acid supplemented states in the plasma fluxes of leucine and α-ketoisocaproate and the rates of oxidation of leucine are in accord with these expected from previous work (Castellino *et al*, 1987; Tessari *et al*, 1987). The most important feature of the results for the present discussion is the net anabolic effect of exogenous amino acids due to an increase in non-oxidative leucine disappearance (whole body protein synthesis) and a reduction in the appearance of leucine from protein breakdown.

Given the fact that skeletal muscle contributes about 50% of lean body mass (Forbes, 1987) it is possible to calculate (assuming that muscle contains 180 mg protein/g, protein contains 8% leucine and all skeletal muscle is similar to anterior tibialis) the contribution of muscle to whole body protein synthesis (non-oxidised leucine disappearance); it would be 24 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ in the postabsorptive state and 33 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ during exogenous amino acid infusion. These estimates would be reduced by 16% if skeletal muscle turnover generally reflected that of quadriceps. Such calculations suggest that in the postabsorptive state skeletal muscle may account for between 20–24% of whole body protein synthesis. The relative change, on addition of exogenous amino acids, was twice as great for anterior tibialis than for the whole body protein mass suggesting that muscle increased its contribution; indeed calculations show that the proportion of whole body protein synthesis due to muscle rose to between 23-28%. This is less than the

contribution previously calculated (Rennie *et al*, 1982b) because of the revised, lower values now routinely obtained for skeletal muscle protein synthesis (Gibson *et al*, 1987; Halliday *et al*, 1988).

This work provides strong support for the hypothesis that amino acids have an anabolic effect by stimulating muscle protein synthesis. This effect of amino acids may explain why insulin *per se* fails increase muscle protein synthesis as reductions occur in amino acid concentration in plasma (Fukagawa *et al*, 1986) and muscle (Alvestrand *et al*, 1988) during hyperinsulinaemia. However, uncertainties regarding the correct accessible leucine metabolite to use to define precursor pool enrichment remain to be resolved. The biopsy technique provides no information about skeletal muscle protein breakdown or intermediary metabolism of leucine by the leg. To investigate the effect of amino acids on these aspects of muscle protein turnover and amino acid metabolism further studies assessing arteriovenous exchange of tracers (Sections 2.6 and 2.7) are indicated (Chapter 5).

Chapter 5. EFFECT OF AMINO ACID INFUSION ON LEG PROTEIN TURNOVER IN HEALTHY SUBJECTS ASSESSED BY [^{15}N]PHENYLALANINE AND [1-^{13}C]LEUCINE EXCHANGE

CONTENTS

TABLES

FIGURES

5.1 Summary

A stable isotope technique depending on the use of [^{15}N]phenylalanine and [1-^{13}C]leucine to assess leg exchange of the tracee amino acids was utilized to measure the components of protein turnover of the human leg and the effects of exogenous amino acid supply. Eight healthy subjects were studied when postabsorptive in the basal state and again during infusion of a mixed amino acid solution (55 g/l; 1.52 ml•kg^{-1}•h^{-1}). During the basal period leucine oxidation by the leg was 4.4±2.0 nmol•100 g^{-1}•min^{-1} and this increased threefold during amino acid infusion (13.6±3.1 nmol•100 g^{-1}•min^{-1}, mean±SEM, P=0.003). Amino acid infusion abolished the net negative balance between incorporation of phenylalanine into, and release from, protein (basal, -13.7±1.8; during infusion, -0.8±3.0 nmol•100 g^{-1}•min^{-1}, P=0.003); leucine exchange showed a similar pattern (basal, -31.8±5.8; during infusion, +3.1±7.1 nmol•100 g^{-1}•min^{-1}, P=0.001). Basal entry of phenylalanine into leg protein (i.e. protein synthesis) was 29.1±4.5 nmol•100 g^{-1}•min^{-1} and this increased during amino acid infusion to 38.3±5.8 nmol•100 g^{-1}•min^{-1} (P=0.09). Leucine entry to protein also increased with amino acid infusion 70.0±10.8 *vs* 87.3±14.1 nmol•100 g^{-1}•min^{-1}, P=0.11). Release from protein of phenylalanine (42.8±4.2 *vs* 39.1±4.2 nmol•100 g^{-1}•min^{-1}, P=0.50) and of leucine (101.8±9.1 *vs* 84.2±9.1 nmol•100 g^{-1}•min^{-1}, P=0.21) was unchanged by amino acid infusion. The results suggest that, in the post-absorptive state in man, infusion of mixed amino acids, without additional energy substrates, reverses negative amino acid balance by a mechanism which includes stimulation of muscle protein synthesis but which does not alter protein breakdown. Interpretation of the results on whole-body protein turnover suggests that the increase in muscle protein

synthesis contributes substantially to the whole-body increase, but the fall in whole-body breakdown with exogenous amino acids is independent of changes in muscle.

5.2 Introduction

We have shown, using a technique involving muscle biopsy and incorporation of [1-^{13}C]leucine, that infusion of a mixed amino acid solution alone increases human skeletal muscle protein synthesis (Chapter 4). We wished to check this result by an independent method and obtain additional information about the effects of amino acid infusion on other components of skeletal muscle protein and amino acid metabolism, principally protein breakdown and leucine oxidation. Investigation of skeletal muscle protein turnover *in vivo* in man has previously been usually studied using tracer incorporation into muscle biopsies (Halliday and McKeran, 1975; Rennie *et al*, 1982b; Gibson *et al*, 1988; Halliday *et al*, 1988; Nair *et al*, 1988b; Gibson *et al*, 1989) but this method measures protein synthesis alone, providing no information on protein breakdown or other metabolic processes.

An alternative approach to amino acid metabolism in human muscle is to measure arteriovenous exchange of leucine across forearm, with L-[1-^{13}C,^{15}N]leucine (Cheng *et al*, 1985; Cheng *et al*, 1987). This technique provides additional information about oxidation, transamination and release from protein of leucine but has the disadvantage of requiring mass spectrometric analysis of ^{15}N and ^{13}C enrichment of different leucine fragments, α-ketoisocaproate and CO_2 and the proliferation of a number of model dependent assumptions. The conceptually similar technique using tracer phenylalanine (Gelfand and Barrett, 1987) has the

advantage of employing fewer (and a different subset of) modelling assumptions and provides a more direct measure of protein breakdown. We have borrowed from and modified these techniques by simultaneously employing a leucine tracer incorporating only one labelled moiety (L–[1–^{13}C]leucine) and a stable isotope-labelled phenylalanine tracer (L–[^{15}N]phenylalanine) in order to provide relatively robust estimates of protein breakdown in human muscle. Concurrent to our study a conceptually similar technique was evolved by a separate group of workers using [*phenyl*–$^{2}H_{2}$]phenylalanine and [*phenyl*–$^{2}H_{4}$]phenylalanine (Thompson *et al*, 1989).

We have used these methods to investigate the effect of infusion of mixed amino acid solution in young healthy subjects. Our aims were (a) to gain a further insight into the possible effects of amino acids alone on human skeletal muscle protein and amino acid metabolism *in vivo* and (b) to compare the results obtained with the arteriovenous exchange technique with those from muscle biopsy studies previously performed under similar conditions, which showed a stimulation of protein synthesis (Chapter 4).

5.3 Methods

5.3.1 Subjects

Eight healthy subjects (2 female, 6 male; age 28.5±2.5 years; weight 72.0±3.6 kg; body mass index 23.5±1.1 kg/m^2) were studied.

5.3.2 Protocol

After an overnight fast (15 h) the subjects were investigated during 180 min of continued fasting (basal), followed by 180 min of infusion of mixed amino acids

(Protocol c, Section 3.10.3, Figure 3.3). Cannulas for blood sampling were placed in a dorsal hand vein, in a retrograde direction, and in a common femoral vein, in an antegrade direction (Section 3.5). A third cannula was placed in a contralateral forearm vein for tracer and amino acid infusion.

Priming doses of L-[1-^{13}C]leucine (6.87±0.20 μmol/kg), L-[^{15}N]phenylalanine (2.75±0.10 μmol/kg) and $NaH^{13}CO_3$ (1.99±0.08 μmol/kg) were administered at time 0 min. A continuous infusion of L-[1-^{13}C]leucine (7.61±0.21 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) and L-[^{15}N]phenylalanine (3.05±0.08 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) was established and continued until the end of the study. After 180 min of continued fasting a priming dose (97.4 mg amino acids/kg over 10 min) of a commercial mixed amino acid solution (Synthamin 9, Section 3.3, Table 3.1) was administered with a second priming dose of $NaH^{13}CO_3$ (0.97±0.04 μmol/kg). Synthamin 9 was then infused until the end of the study at 1.52 $ml \cdot kg^{-1} \cdot h^{-1}$, providing 83.5 $mg \cdot kg^{-1} \cdot h^{-1}$ of mixed amino acids, 46.5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ leucine and 0.33 g $nitrogen \cdot kg^{-1} \cdot day^{-1}$.

Arterialized venous samples were taken before the start of the tracer infusion (-15 min) and during each phase of study (Section 3.5, Figure 3.3). Femoral venous samples and both arterialized and femoral venous blood samples for subsequent determination of ^{13}C enrichment of blood CO_2 (Section 3.5.1) were only taken before the tracer was administered and at the four time points at the end of each phase of study.

Ventilated-hood indirect calorimetry (Section 3.6) was undertaken during the final 40 min of each phase of study. Leg blood flow (Section 3.8) was measured three or four times after each of the last set of four pairs of blood samples during each phase of study.

Table 5.1. Hormones and Substrate Concentrations in Blood and Plasma in the Absence and in the Presence of Amino Acid Infusion

	Basal	Amino acid infusion
Plasma		
Insulin (pmol/l)	48±6	56±8[a]
IGF-I (U/l)	740±80	750±80
Glucagon (pmol/l)	39.4±10	54.0±8.6[b]
Cortisol (nmol/l)	189±32	201±34
Glucose (mmol/l)	4.8±0.1	4.9±0.1
Blood		
Lactate (mmol/l)	0.65±0.08	0.69±0.06
D-(−)-3-Hydroxybutyrate (mmol/l)	0.14±0.03	0.11±0.02

Results are means±SEM. Statistical significance: [a]P=0.07, [b]P<0.01.

IGF-I, Insulin-like growth factor I.

Amino acid concentration analysis was by automated ion exchange chromatography with fluorometric detection (Section 3.13).

5.4 Results

5.4.1 Indirect Calorimetry

Whole-body carbon dioxide production was 0.191±0.011 l/min during basal postabsorptive conditions and this increased during amino acid infusion to 0.208±0.11 l/min ($P<0.01$); whole-body oxygen consumption was 0.247±0.014 l/min and also increased during amino acid infusion to 0.275±0.017 l/min ($P<0.01$); thus resting energy expenditure was 4.96±0.28 kJ/min during basal conditions and increased during amino acid infusion to 5.51±0.34 kJ/min ($P<0.01$). Leg blood flow was 2.44±0.23 ml•100 g^{-1}•min^{-1} during the basal phase of study and there was no change during mixed amino acid infusion (2.69±0.18 ml•100 g^{-1}•min^{-1}).

5.4.2 Hormones and Substrates

The basal plasma glucagon concentration (Table 5.1) was 39.4±10 pmol/l and increased by 37% during mixed amino acid infusion ($P<0.01$). The basal plasma insulin concentration was 48±6 pmol/l and increased by 14% during amino acid infusion ($P=0.07$, one-tailed). The concentrations of plasma insulin-like growth factor I, cortisol, glucose, blood lactate and D-(-)-3-hydroxybutyrate remained unaffected by the amino acid infusion.

5.4.3 Amino Acid Arterial Concentrations

The primed amino acid infusion produced a rapid increase in the arterial plasma concentration of most amino acids (Table 5.2). The plasma concentrations of phenylalanine and leucine (Figure 5.1) were stable during each phase of study. Phenylalanine concentration increased by a mean of 91%, leucine by a mean of

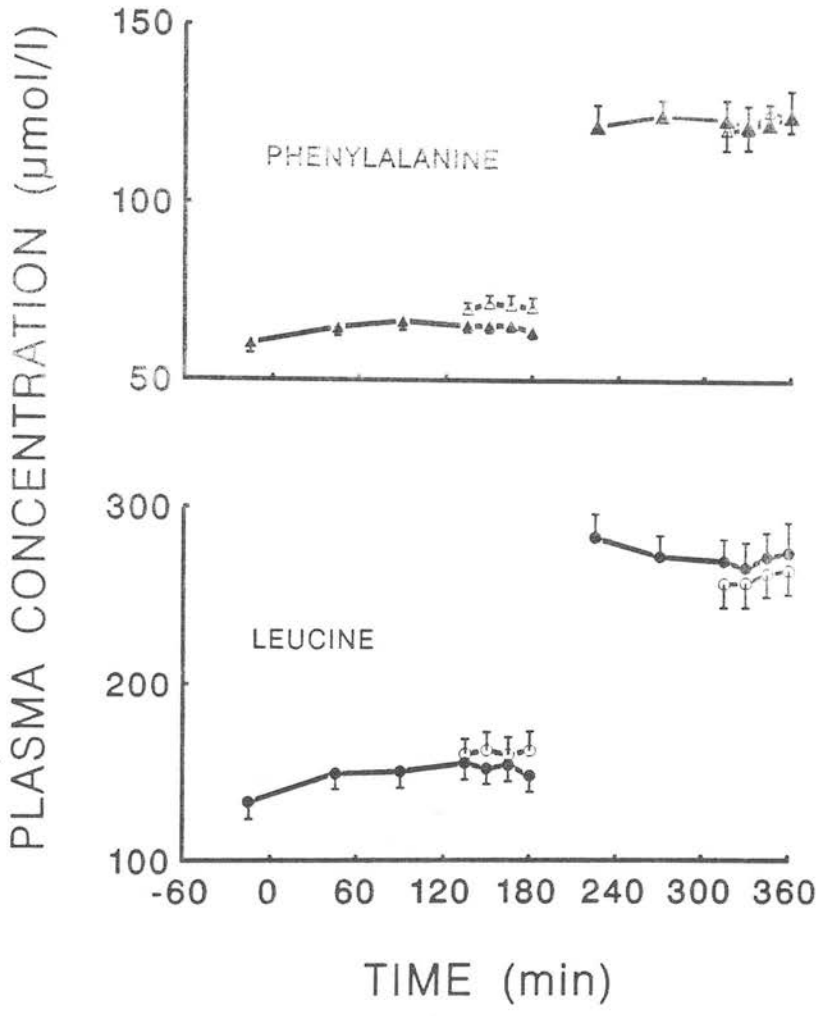

Figure 5.1. Plasma phenylalanine and leucine concentrations before (-15 to 180 min) and in the presence (180 to 360 min) of mixed amino acid infusion (solid symbols, arterial; hollow symbols, venous)

77% and total amino acids by a mean of 57% during the amino acid infusion. A reduction occurred in the arterial concentration of 3-methylhistidine and there was a slight increase in the concentration of glutamine during the amino acid infusion.

Table 5.2. Arterial Plasma Amino Acid and α-Ketoisocaproate Concentrations

	Basal (μmol/l)	Amino acid infusion (μmol/l)
Alanine	237±16	526±26[c]
Glutamate	70.3±10.1	74.7±9.8[a]
Glutamine	547±37	578±44
α-Ketoisocaproate	33.6±4.0	36.3±4.0[b]
3-Methylhistidine	3.99±0.27	3.70±0.32[a]
Isoleucine	54±4	166±9[c]
Leucine	153±9	271±14[c]
Phenylalanine	65±2	124±6[c]
Tyrosine	48.4±3.6	52.9±4.4[b]
Valine	212±15	363±19[c]
BCAA	418±27	801±40[c]
Total	2630±90	4140±170[c]

Statistical significance: [a]$P<0.05$, [b]$P<0.02$, [c]$P<0.001$.

BCAA, branched-chain amino acids.

5.4.4 Leg Amino Acid Balance

The net effluxes from the leg of both phenylalanine and tyrosine (Table 5.3) lessened but remained negative during amino acid infusion, indicating that leg protein breakdown still exceeded protein synthesis. The efflux of 3-methylhistidine from the leg was unchanged throughout suggesting that the rate of myofibrillar protein breakdown was unaffected by amino acid infusion.

5.4.5 Enrichments of Phenylalanine, Leucine and Leucine Metabolites

Plateaux were attained for the enrichments of plasma phenylalanine, leucine (Figure 5.2) and α-ketoisocaproate and both whole-blood and expired CO_2 during the final periods of each phase of study.

5.4.6 Leg Protein Turnover

Leg mixed protein breakdown, assessed with either [^{15}N]phenylalanine or [1-^{13}C]leucine, (Table 5.4) was not altered by infusion of amino acids. Leg protein synthesis assessed with phenylalanine (Figure 5.3) increased during infusion of amino acid 32% (P=0.09, one-tailed; 95% confidence interval -5.2 to +23.7 nmol•100 g^{-1}•min^{-1}). Leg protein synthesis, measured with leucine tracer, showed a mean value which was 25% greater during infusion of mixed amino acids (P=0.11, one-tailed; 95% confidence interval -15.2 to +49.6 nmol•100 g^{-1}•min^{-1}). Oxidation of leucine by the leg increased 3 fold (P<0.01). The balance between protein synthesis and protein breakdown became markedly (P<0.01) less negative during infusion of amino acids when assessed by both phenylalanine and leucine tracer kinetics. The molar ratio of leucine to phenylalanine entry to leg protein was

2.4 when the subjects were studied during postabsorptive conditions and was 2.3 when they were infused with amino acids; in skeletal muscle protein the ratio of the concentrations of the amino acids is 2.4 (Gelfand and Barrett, 1987).

5.4.7 Whole-Body Leucine and Phenylalanine Kinetics

Whole-body leucine flux (Table 5.5) increased by 24% during the amino acid infusion and phenylalanine flux increased by 34%. Assessment of whole-body protein breakdown with leucine tracer suggested that it decreased by 14% during the amino acid infusion; assessment with phenylalanine tracer suggested a decrease of 23%. The molar ratio of leucine to phenylalanine release from protein was 2.4 (basal) and 2.6 during amino acid infusion. Whole-body oxidation of leucine almost doubled and whole-body protein synthesis assessed with leucine increased by 12% ($P<0.02$) during infusion of mixed amino acids. The balance between protein synthesis and protein breakdown in the whole-body, assessed with leucine, changed from negative to positive, a highly significant result ($P<0.01$).

5.4.8 Calculated Leg Muscle Fractional Protein Synthetic Rate

With the information that muscle contains 18% protein, protein contains 8% leucine and 4.0% phenylalanine (Gelfand and Barrett, 1987), and assuming (on the fat content of the subjects, and that the leg is representative of whole-body) that 75±3% of leg blood flow is distributed to muscle, the basal fractional protein synthetic rate may be calculated as 0.052±0.009 %/h, from phenylalanine exchange, or 0.052±0.009 %/h, from leucine exchange. The corresponding rates during amino acid infusion were 0.074±0.015 %/h and 0.066±0.012 %/h respectively.

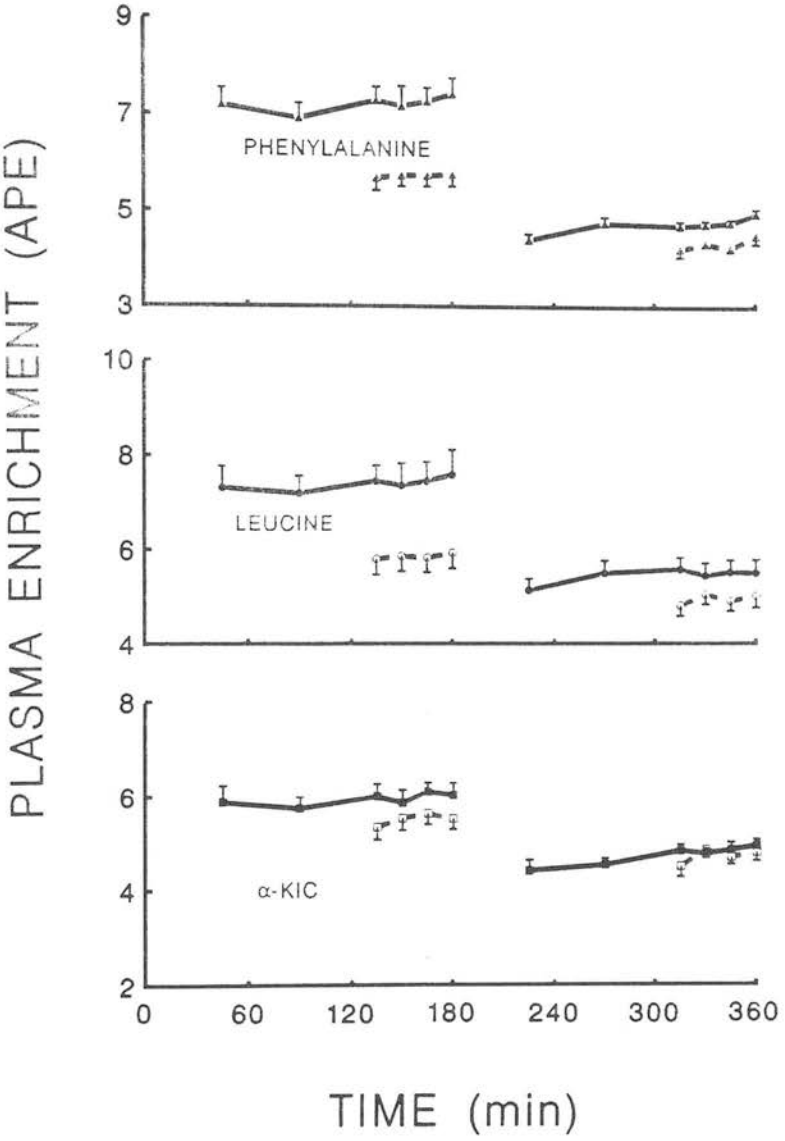

Figure 5.2. Plasma phenylalanine, leucine and α-ketoisocaproate enrichments in the absence and in the presence of mixed amino acid infusion (solid symbols, arterial; hollow symbols, venous)

5.5 Discussion

During the investigations described above we were able to produce, by infusion, a 60% increase in the plasma concentrations of total amino acids; all indispensable amino acids increased in concentration. Thus we were in a position to compare metabolism with and without the presence of extra amino acids. Both plasma glucagon and insulin concentrations increased slightly (16 and 14%, respectively) but the other hormones and substrates monitored were not altered in concentration by the infusion. Thus the factor most likely to have any major anabolic effect would be the large change in amino acid concentration. However the most striking effect of the amino acid infusion on leg amino acid metabolism was a catabolic one, a three fold increase in leucine oxidation. This is likely to have been the result of stimulation secondary to the 77% increase in leucine concentration and a subsequent increase in α–ketoisocaproate, its keto acid, either by mass action or by activation of intra-mitochondrial branched-chain keto acid dehydrogenase complex (Section 2.3.1; Khatra *et al*, 1977; May and Buse, 1989). (It has been suggested from studies in man (Nair *et al*; 1988b) and in pig (Ostaszewski and Nissen, 1988) that glucagon at high concentrations stimulates whole-body leucine oxidation, and this may be an additional factor which increased leucine oxidation by skeletal muscle and other tissues in the present study). The net efflux of α–ketoisocaproate from the leg was 26% higher during the amino acid infusion showing that net transamination of leucine was increased more than decarboxylation by the amino acid infusion. However the present results confirm and extend previous findings that the majority of leucine transaminated in muscle

is oxidised *in situ* and not exported to liver as α-ketoisocaproate as in the rat (Elia and Livesey, 1983).

The unidirectional uptake by the leg of both leucine and of phenylalanine (i.e. protein synthesis) was increased by the infusion of amino acids. A substantial degree of variation in the measured response to the infusion was seen in the different individuals, due both to analytical and biological factors. Nevertheless the finding that the average leg protein synthetic rate increased in response to amino acid infusion is in agreement with the results from a previous study in which protein synthetic rate, indicated by incorporation of tracer into percutaneous muscle biopsies, was found to increase by 35% (Chapter 4).

The modest increase in insulin during amino acid infusion may theoretically have stimulated skeletal muscle protein synthesis, although in adult man no such effect of insulin alone on skeletal muscle protein synthesis could be found by techniques similar to these used here (Gelfand and Barrett, 1987). In contrast we found an effect of insulin on muscle protein synthesis assessed by phenylalanine arteriovenous exchange at fast rates of amino acid supply (Chapter 6), but we have evidence that in diabetic man protein synthesis studied by the biopsy incorporation technique is unaffected by acute insulin in the presence of amino acids (Chapter 7). We previously concluded that the rise in protein synthesis observed during amino acid infusion was either a direct effect of the increased amino acid availability (Chapter 4), or due to increases in an unidentified systemic or paracrine growth factor secondary to increased amino acid concentration.

It is possible that the increase in the plasma concentrations of both leucine and phenylalanine which occurred during amino acid infusion may have caused an

Table 5.3. Leg Net Balance of Amino Acids and α-Ketoisocaproate; Effects of Amino Acid Infusion

	Basal	Amino acid infusion
	(nmol•100 g^{-1}•min^{-1})	
Alanine	-145±13	-45±16[c]
Glutamate	83±11	101±11
Glutamine	-196±18	-221±27
α-Ketoisocaproate	-6.9±3.6	-8.7±3.1
3-Methylhistidine	-0.67±0.17	-0.62±0.15
Isoleucine	-13.6±1.4	21.2±5.0[c]
Leucine	-20.6±3.9	25.4±6.8[c]
Phenylalanine	-13.8±1.8	-0.8±2.9[b]
Tyrosine	-12.7±1.3	-7.0±1.5[a]
Valine	-21.6±5.0	28.4±6.3[c]
BCAA	-55.8±8.2	75.1±17.6[c]
Total	-480±44	-159±118[b]

Statistical significance: [a]$P<0.02$, [b]$P<0.01$, [c]$P<0.001$.

BCAA, Branched chain amino acids.

increase in tracer exchange between the plasma and intramuscular compartments

(Layman and Wolfe, 1987). If during basal conditions tracer exchange between plasma and intracellular fluid was incomplete and a marked difference existed between the intracellular tracee enrichment and that of plasma, such a difference would be less when amino acid concentrations were raised by exogenous infusion and amino acid transport was stimulated. Consequently protein breakdown and protein synthesis may have been significantly underestimated during basal conditions but underestimated to a lesser extent during mixed amino acid infusion. If this potential source of error had occurred to a marked extent the present results may overestimate the stimulatory effect of amino acid infusion on protein synthesis.

Apart from the measurements of net transamination and oxidation we have more confidence in the results for protein metabolism derived from phenylalanine than leucine data. This is for a number of reasons. For the leucine-based results (e.g. the derived leg protein synthesis value calculated from 14 separate analyses: blood flow, packed cell volume, arterial and venous concentrations and enrichments of leucine, α–ketoisocaproate and CO_2) the analytical variation in the calculated protein synthetic rate was greater than when phenylalanine data, for which only 6 separate analyses were required, were used. A further potential problem with the leucine-based results is the possibility that the use of plasma α–ketoisocaproate as a measure of the precursor pool enrichment for leucine oxidation and the weighted mean of leucine and α–ketoisocaproate as the precursor pool for protein synthesis may cause a systematic error in the leucine-based results if one of these estimates of precursor pool enrichment is incorrect. There is also the possibility of a systematic error in the calculation of leucine oxidation which may occur to a

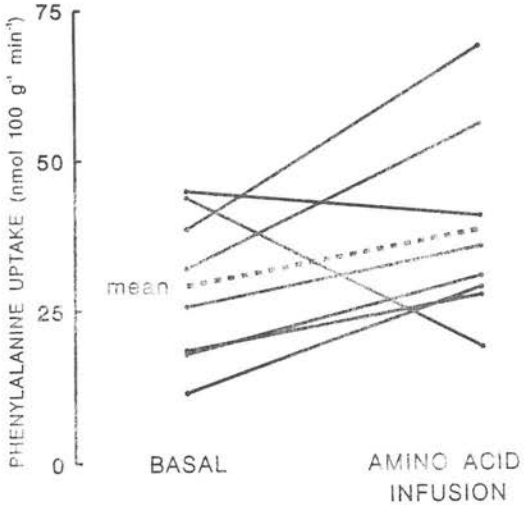

Figure 5.3. Leg protein synthesis before and during mixed amino acid infusion assessed with phenylalanine exchange

substantial extent in leg adipose tissue (Frick and Goodman, 1980) in addition to muscle, whereas protein synthesis will occur predominantly in muscle. For phenylalanine similar problems do not arise as adipose tissue is not a significant site for its metabolism. It therefore appears likely that the interpretation of the results of leg protein turnover measured with phenylalanine in an exchange model may be more secure than those obtained from measures with leucine. Although the leucine technique, particularly when a L–[1–^{13}C,^{15}N]leucine tracer is used (Cheng *et al*, 1985; Cheng *et al*, 1987) potentially provides additional information about amino acid intermediary-metabolism (e.g. unidirectional transamination rates) by the leg, the stable isotope-labelled phenylalanine technique is also more analytically

robust, obviating the need for isotope ratio mass spectrometry of CO_2 and providing more secure information.

Table 5.4. Components of Leg Phenylalanine and Leucine Metabolism; Effects of Amino Acid Infusion

	Phenylalanine turnover		Leucine turnover	
	Basal	Amino acid infusion	Basal	Amino acid infusion
	(nmol•100 g^{-1}•min^{-1})		(nmol•100 g^{-1}•min^{-1})	
Breakdown	42.8±4.2	39.1±4.2	101.8±9.1	84.2±9.1
Synthesis	29.1±4.5	38.3±5.8[b]	70.0±10.8	87.3±14.1[a]
Oxidation	--	--	4.4±2.0	13.6±3.1[c]
Balance (Synthesis-Breakdown)	-13.8±1.8	-0.8±2.9[c]	-31.8±5.8	3.1±7.1[d]

Statistical significance: [a]P=0.12, [b]P=0.09, [c]P<0.01, [d]P<0.001.

When amino acids are administered intravenously in the form of a primed continuous infusion the intravenous amino acid pool expands rapidly and a new plateau for amino acid concentration may be obtained within 45 min (Figure 5.1). However, the intramuscular amino acid pool may expand in size more slowly, being limited by the transport kinetics of amino acids. Studies of the effect of amino acid

infusion for only 90 min (Lundholm *et al*, 1987) suggest that equilibration of concentration between plasma and muscle is not totally complete within this short period although when amino acids were infused for 240 min equilibration did seem to occur (Chapter 4). Such observations suggest that disappearance from the plasma space of non-oxidised/non-transaminated leucine carbon and of phenylalanine into leg skeletal muscle after 135-180 min of amino acid infusion predominantly represents incorporation of the amino acid into protein. Nevertheless we cannot exclude the possibility that tracer uptake across the leg may in part represent equilibration of the amino acids between plasma and muscle on expansion of the intramuscular free amino acid pool and, consequently, protein synthesis may have been overestimated during amino acid infusion in the present study. However our previous finding of an increase in skeletal muscle protein synthesis, determined by leucine incorporation into protein (Chapter 4), is not dependent on the particular modelling-assumptions adopted in the present work and the agreement of the of the two results suggests that the design of the present study was adequate. An additional piece of evidence providing support for the nature of the results is the concurrence between the ratios of influx and efflux of tracer leucine and phenylalanine and their relative concentrations in muscle protein.

The effects of amino acid infusion on whole-body protein turnover determined with leucine are in agreement with our previous study (Chapter 4) which showed increases in whole-body protein synthesis and oxidation, a reduction in whole-body protein breakdown, and a change in whole-body protein balance from negative to positive. The reduction in whole-body protein breakdown during

Table 5.5. Components of Whole-Body Leucine and Phenylalanine Kinetics; Effects of Amino Acid Infusion

	Leucine turnover		Phenylalanine turnover	
	Basal	Amino acid infusion	Basal	Amino a infusion
	($\mu mol \cdot kg^{-1} \cdot h^{-1}$)		($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	
Flux	119±3	148±5[c]	50.0±2.1	66.9±2.6[b]
Infusion	--	46	--	28.2
Breakdown	119±3	102±5[b]	50.0±2.1	38.7±2.6[b]
Oxidation	19±1	37±2[c]		
Synthesis	100±2	112±4[a]		
Balance (Synthesis-Breakdown)	-19±1	10±2[c]		

Statistical significance: [a]$P<0.02$, [b]$P<0.01$, [c]$P<0.001$.

amino acid infusion was confirmed by the phenylalanine-based data.

The present investigation suggests that infusion of mixed amino acids neither decreased total mixed skeletal muscle protein breakdown (indicated by the leucine and phenylalanine results) or reduced myofibrillar protein breakdown

(indicated by the lack of change in 3-methylhistidine efflux), findings in agreement with those from a previous study (Tracey *et al*, 1988). Thus the decrease in whole-body protein breakdown must occur in non-skeletal muscle tissue, such as the liver, a tissue in which protein breakdown is inhibited by amino acid availability (Mortimore *et al*, 1987).

The calculated fractional leg skeletal muscle protein synthetic rates for postabsorptive subjects (phenylalanine-based 0.052±0.009 %/h; leucine-based 0.052±0.009 %/h) are very close to these for anterior tibial muscle determined in biopsy samples and calculated with plasma α-ketoisocaproate as precursor pool for protein synthesis (0.055±0.003 %/h, Chapter 4). However, the rates for anterior tibialis, when calculated with α-ketoisocaproate as precursor pool (Chapter 4), are about 20% higher than the rates for quadriceps muscle (Gibson *et al*, 1987) measured with identical means; the rates from quadriceps will be more representative of the average skeletal muscle protein synthetic rates of the whole-leg than anterior tibialis. The rates obtained with the arteriovenous exchange technique should be viewed as minimal estimates of muscle protein synthesis since the use of the plasma amino acid tracer enrichment in the calculations may result in an overestimate of the enrichment of the precursor pool for protein synthesis and correspondingly underestimate protein synthesis.

Nevertheless, the results provide further evidence that nutritionally driven anabolism in the whole-body receives a major input from skeletal muscle. The present study confirms our previous findings that infusion of mixed amino acid solution without addition hormones or energy substrates increases skeletal muscle protein synthesis and leucine transamination and oxidation. In contrast, it also

suggests that amino acid infusion does not alter either mixed protein breakdown or myofibrillar protein breakdown of skeletal muscle of the leg. A major part of the anabolic effect of feeding, in the whole-body, may be due simply to an increased supply of amino acids on muscle protein synthesis, but the decrease in whole-body protein breakdown must occur in non-muscle tissue.

Chapter 6. STIMULATION OF SKELETAL MUSCLE AND WHOLE BODY-PROTEIN SYNTHESIS IN HEALTHY SUBJECTS BY INSULIN: LEG EXCHANGE AND PLASMA KINETICS DURING AMINO ACID INFUSION AND EUGLYCAEMIC HYPERINSULINAEMIA

CONTENTS

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FIGURES

6.1 Summary

Leg exchange and plasma kinetics of [^{15}N]phenylalanine and [1-^{13}C]leucine were used to assess the effect of insulin during amino acid sufficiency on leg and whole-body protein turnover. Eight healthy subjects were studied first during amino infusion alone and then during infusion of glucose and insulin (0.29 nmol•m^{-2}•min^{-1}) with additional amino acids. Insulin strongly stimulated the positive leg amino acid balance seen with amino acids; phenylalanine uptake into leg protein (protein synthesis) rose during insulin plus amino acids (amino acids alone, 47.3±11.5 nmol•(100 g leg)$^{-1}$•min^{-1}; insulin + amino acids, 73.1±7.3 nmol•100 g^{-1}•min^{-1}, P=0.022) but with no reduction in leg phenylalanine release (protein breakdown) (44.7±8.1 *vs* 40.0±7.9; both nmol•100 g^{-1}•min^{-1}). Leucine entry to leg protein increased slightly with insulin (129±26 *vs* 146±21 nmol•100 g^{-1}•min^{-1}) possibly obscured by a fourfold elevation in leucine oxidation (P=0.012). Leg protein breakdown (leucine release) was reduced by insulin (120±17 *vs* 84±10 nmol•100 g^{-1}•min^{-1}, P=0.024). Whole-body protein synthesis (non-oxidative leucine-carbon disposal) increased with insulin (114±4 *vs* 126±4 μmol•kg^{-1}•h^{-1}, P=0.0048); whole-body protein breakdown (as appearance of either endogenous leucine or phenylalanine) fell with insulin (leucine, 118±5 *vs* 99±6; phenylalanine, 45±2 *vs* 32±5; both μmol•kg^{-1}•h^{-1}, both P<0.02). The results suggest that insulin, given with sufficient amino acids, markedly stimulates leg and whole-body protein balance by mechanisms including stimulation of protein synthesis and inhibition of protein breakdown.

6.2 Introduction

Insulin is well recognised to exert a protein anabolic effect in man, the anabolic effect probably including modulation of both protein synthesis and breakdown. A stimulatory effect on protein synthesis has been shown in animal tissues both *in vitro* and *in vivo* (Section 1.8.1), but the post-receptor mechanisms of insulin stimulation of protein synthesis have not been fully elucidated (Kahn and White, 1988). Studies of animal tissue extracts *in vitro* suggest that insulin deficiency may cause impaired transcription, leading to a decrease in mRNA production (Dillmann, 1988) and impaired initiation of translation due to a reduction in the proportion of total ribosomes as polyribosomes (Section 1.8.1; Kimball and Jefferson, 1988). Insulin also stabilizes lysosomes and decreases rat muscle (Furuno and Goldberg, 1986) and liver protein breakdown (Mortimore *et al*, 1987) *in vitro* (Kettelhut *et al*, 1988), but is not generally considered to affect the rate of myofibrillar protein breakdown (Smith and Sugden, 1986), a cytoplasmic rather than lysosomal process (Furuno and Goldberg, 1986).

Despite the readily apparent stimulatory effect of insulin on protein synthesis in animal tissues both *in vivo* and *in vitro*, demonstration of such an effect *in vivo* in man has proved elusive, either for muscle (Pacy *et al*, 1989), or in the whole-body (Castellino *et al*, 1987; Tessari *et al*, 1987). Furthermore, in those studies where arterial amino acid concentrations were maintained at post-absorptive values, with insulinaemia the major variable, no stimulatory effect of insulin on protein synthesis was apparent, either for forearm (Gelfand and Barrett, 1987) or whole-body (Castellino *et al*, 1987; Tessari *et al*, 1987). One explanation for the apparent failure of insulin to stimulate protein synthesis is a possible

constraint caused by an insulin-induced reduction in amino acid concentrations both in plasma and in muscle (Alvestrand *et al*, 1988). The importance of prevailing amino acid concentration is demonstrated by our findings, by two independent methods, that increased amino acid availability alone stimulates skeletal muscle as well as whole-body protein synthesis in healthy young subjects (Chapters 4 and 5).

The present studies were undertaken to assess the acute effects of insulin on protein metabolism both of skeletal muscle and of the whole-body in healthy subjects under conditions of amino acid replacement designed to provide a sufficiency of amino acids during periods of physiologically low and high insulinaemia.

6.3 Methods

6.3.1 Subjects

Eight healthy subjects (2 female, 6 male; mean age 33, range 24-42 years, weight 68.7±3.9 kg; body mass index 23.6±1.3 kg/m^2) with no intercurrent or chronic illness and with normal hepatic, renal and thyroid function were studied. Three additional male subjects (age 29-30 years, weight 72.0±3.0 kg, body mass index 24.3±1.5 kg/m^2) were studied without tracer (see below).

6.3.2 Protocol

After an overnight fast (15 h), from 08-30 h the subjects received only water and no food by mouth until the study was complete. Their leg and whole-body

Table 6.1. Hormones and Substrate Concentrations in Plasma and Blood; Effects of Insulin During Amino Acid Infusion

	Amino Acids Alone	Insulin plus Amino acids
Plasma		
Insulin (pmol/l)	57±7	597±44[c]
IGF-I (U/l)	650±80	630±80
Glucagon (pmol/l)	34±3	49±8[a]
Cortisol (nmol/l)	167±44	242±56
Glucose (mmol/l)	5.1±0.1	4.9±0.1
Blood		
Lactate (mmol/l)	0.54±0.03	0.91±0.07[c]
D-(-)-3-Hydroxybutyrate (mmol/l)	0.15±0.04	0.05±0.01[b]

Statistical significance: [a]P=0.08, [b]P<0.05, [c]P<0.001; IGF-I, Insulin-like growth factor I.

phenylalanine and leucine kinetics were investigated during 180 min with amino acid, but no insulin, infusion (amino acids alone), followed by 180 min of infusion of glucose and insulin to produce euglycaemic hyperinsulinaemia (the insulin infusion period) (Protocol d, Section 3.10.4, Figure 3.4). They were continuously

infused with the mixed amino acid solution (Synthamin 14, Section 3.3, Table 3.1) throughout, but at an increased rate during insulin infusion, estimated from our experience to maintain a sufficiency of amino acids, in plasma and in muscle, during both phases of study (Chapter 7).

Cannulas for blood sampling was placed in a dorsal hand vein, in a retrograde direction, and in a common femoral vein, in an antegrade direction (Section 3.5). Before sampling arterialized venous blood, the hand was placed for a minimum of 15 min in a temperature-regulated (75-80°C) chamber. A third cannula was placed in a contralateral forearm vein for tracer and amino acid infusions.

Priming doses of L–[^{15}N]phenylalanine (3.06±0.13 μmol/kg), L–[1–^{13}C]leucine (7.55±0.28 μmol/kg) and $NaH^{13}CO_3$ (3.06±0.32 μmol/kg) were administered and a continuous infusion of a mixture of L–[^{15}N]phenylalanine (3.38±0.12 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) and L–[1–^{13}C]leucine (8.28±0.30 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) was established and continued until the end of the study. A priming dose (51.6 mg amino acids/kg over 10 min) of the commercial amino acid solution was administered at the beginning of the study; it was then infused until the end of the basal period at a rate of 0.52 $ml \cdot kg^{-1} \cdot h^{-1}$, providing 44.2 $mg \cdot kg^{-1} \cdot h^{-1}$ of mixed amino acids, 15.0 μmol phenylalanine$\cdot kg^{-1} \cdot h^{-1}$, 24.6 μmol leucine$\cdot kg^{-1} \cdot h^{-1}$ and 0.17 g nitrogen$\cdot kg^{-1} \cdot day^{-1}$. The subjects were studied over 180 min (amino acids alone) and then during a primed intravenous infusion of neutral human insulin (Humulin S, Eli Lilly and Co. Ltd, Basingstoke, U.K.) of 0.29 $nmol \cdot m^{-2} \cdot min^{-1}$ (40 $mU \cdot m^{-2} \cdot min^{-1}$) for 180 min (the insulin infusion period) (Section 3.9.2). Glucose solution (1 mol/l) was infused at varying to maintain euglycaemia (Section 3.3). During the insulin infusion period,

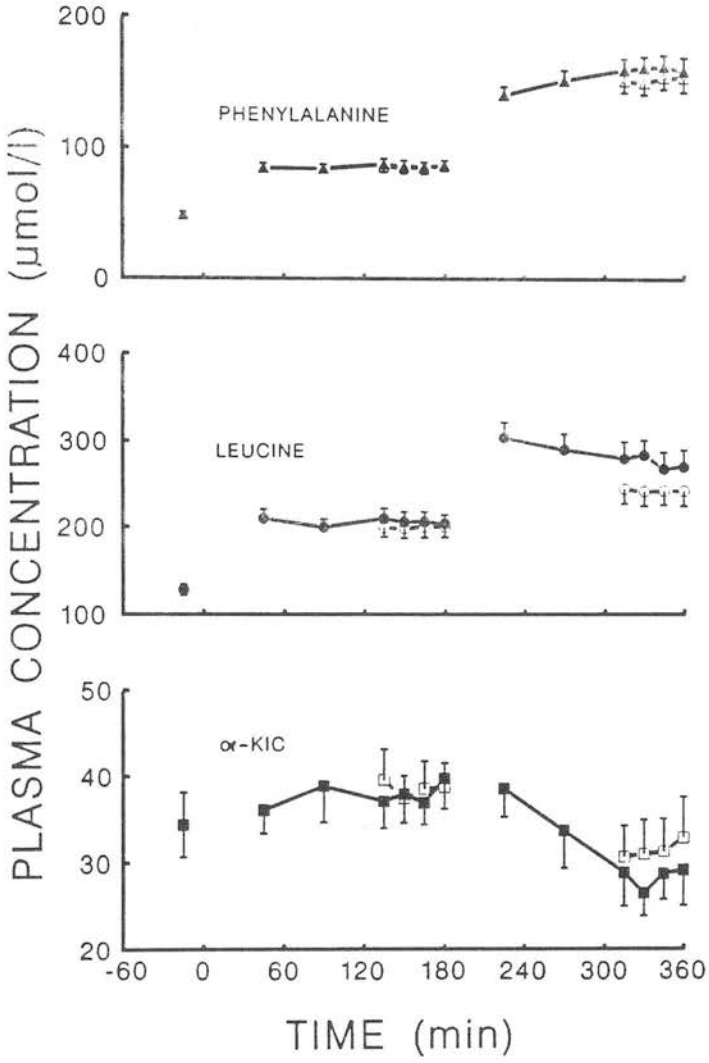

Figure 6.1. Arterial (solid symbols) and venous (hollow symbols) phenylalanine, leucine and α-ketoisocaproate concentrations before and during insulin infusion with continuous amino acid infusion

the amino acid infusion rate was increased to 1.96 $ml \cdot kg^{-1} \cdot h^{-1}$ providing 167 $mg \cdot kg^{-1} \cdot h^{-1}$ of mixed amino acids, 56.5 µmol $phenylalanine \cdot kg^{-1} \cdot h^{-1}$ and 92.7

μmol leucine•kg^{-1}•h^{-1}. The amino acid infusion rates were chosen following a separate set of studies in eight diabetic patients in whom intracellular amino acid concentrations were measured by percutaneous biopsy after 4 h periods of insulin infusion and amino acid infusion at identical rates to these used in this study; in those studies the intramuscular free leucine and total free amino acid concentrations were depressed below normal during insulin infusion even though the plasma amino acids were greater than those during insulin deficiency (Chapter 7). We did not feel justified, given the extensive nature of the study, to carry out muscle biopsies to check the intramuscular concentrations of amino acids in this study.

Arterialized venous samples were taken before the start of the tracer infusion, at intermediate times (45, 90 min) and at the end (135, 150, 165, 180 min) of the first phase of study, and at intermediate times (225, 270 min) and at the end (315, 330, 345, 360 min) of the second phase of study. Femoral venous samples and blood samples for subsequent determination of ^{13}C enrichment and total concentration of blood CO_2 were taken only before the tracer was administered and at the four time points at the end of each phase of study. Arterialized blood for hormone analysis was obtained on two occasions during the final phases of each study.

At each sampling point, expired air was collected into evacuated glass tubes for storage prior to ^{13}C analysis of CO_2 (Section 3.6). Gas exchange was determined during the final 40 min of each phase of study with a ventilated-hood indirect calorimeter (Section 3.6). Leg blood flow (BF) was measured three or four times after each of the last four blood samples during each phase of study (Section 3.8).

Three additional subjects did not receive tracer amino acids; samples were only taken during the insulin infusion period to determine the effect of changes in substrate oxidation during euglycaemic-hyperinsulinaemia on the background rates of $^{13}CO_2$ production by the leg and the whole-body.

Amino acid concentrations in extracts of plasma were determined with an automated amino acid analyser using ion-exchange chromatography for separation and elution of amino acids, derivatization with ninhydrin and photometric detection for quantification (Section 3.13).

The average rate for all muscle in the leg of muscle was calculated from the phenylalanine and leucine based entry rates on the basis that muscle contains 18% protein, protein contains 4% phenylalanine and 8% leucine (Gelfand and Barrett, 1987) and that bone, skin and fat accounted for negligible protein turnover and sampled blood flow. Bone was assumed to occupy 10% of leg volume and whole-body fat composition was taken as representative of that of the leg (21.5±3.3% in the subjects studied, which compares well with a reported value of 17.9% for thighs in sedentary males (Häggmark *et al*, 1978).

6.4 Results

6.4.1 Gas Exchange and Leg Blood Flow

Whole-body carbon dioxide production was 0.185±0.008 l/min during basal conditions and this increased during insulin infusion (0.236±0.13 l/min, $P<0.001$); whole-body oxygen consumption was 0.245±0.012 l/min and increased during insulin infusion (0.281±0.012 l/min, $P=0.003$); the respiratory quotient increased (0.76±0.02

Table 6.2. Arterial Plasma Amino Acid and α–Ketoisocaproate Concentrations; Effects of Insulin During Amino Acid Infusion

	Pre-study	Amino Acids Alone (μmol/l)	Insulin plus Amino Acids
Alanine	275±32	420±28[d]	800±43[d]
Glutamate	61±6	59±6	46±6[b]
Glutamine	591±26	622±26[a]	527±23[d]
α–Ketoisocaproate	35±4	38±3	28±3[a]
Isoleucine	51±5	105±15[d]	180±15[d]
Leucine	128±7	207±11[d]	276±18[c]
Phenylalanine	49±2	87±4[d]	161±9[d]
Tyrosine	62±5	59±4	51±3[a]
Valine	231±13	302±13[d]	438±26[d]
BCAA	410±22	613±24[d]	893±57[d]
Total	2780±80	3830±100[d]	5280±250[d]

Statistical significance: [a]$P<0.05$, [b]$P<0.02$, [c]$P<0.01$, [d]$P<0.001$.

BCAA, Branched chain amino acid.

Comparisons are amino acids alone *vs* pre-study, and insulin-plus-amino acids *vs* amino acids alone.

vs 0.84±0.02, P=0.003). Resting energy expenditure calculated from gas exchange was 4.90±0.23 kJ/min during the low insulin condition and increased 20% during insulin infusion (5.74±0.26 kJ/min, P<0.001). Leg blood flow was 2.95±0.29 ml•100 g^{-1}•min^{-1} during the low insulin period and increased 60% with insulin infusion (4.74±0.54 ml•100 g^{-1}•min^{-1}, P=0.003).

6.4.2 Hormones and Substrates

The plasma insulin concentration of 57±7 pmol/l during infusion of amino acids alone and increased (P<0.001) to 597±44 pmol/l during insulin infusion (Table 6.1). Plasma glucagon concentration was 34±3 pmol/l in the low insulin phase and increased by 50% during insulin infusion (P=0.084). The concentrations of plasma insulin-like growth factor I and cortisol were unaffected by the insulin infusion and the arterial plasma glucose concentration was stable (5.0±0.1 mmol/l) throughout the study. The glucose infusion rate was increased over the period of the insulin infusion reaching 36.1±4.6 μmol•kg^{-1}•min^{-1} during the final sampling period. Uptake of glucose by the leg increased during insulin infusion (0.07±0.10 *vs* 6.08±0.93 μmol•100 g^{-1}•min^{-1}, P<0.001). Blood lactate concentration increased by 68% during insulin infusion (P<0.001) but no change occurred in leg lactate balance (-0.06±0.08 *vs* -0.07±0.10 μmol•100 g^{-1}•min^{-1}). A 67% reduction occurred in blood D-(−)-3-hydroxybutyrate concentration during insulin infusion (P<0.05).

6.4.3 Amino Acid Arterial Concentrations

The primed amino acid infusion during the initial low insulin period produced a 38% increase in the arterial plasma concentration of total amino acids

(Table 6.2). The arterial plasma concentrations of phenylalanine and leucine were stable during this initial phase (Figure 6.1, Table 6.2). During the insulin infusion period, when extra amino acids were infused, phenylalanine concentration initially increased by 85% and remained stable during the sampling period; leucine initially increased by 33% but then showed a slow steady decrease throughout the insulin infusion period (by 3% during the last hour). Reductions also occurred in the arterial concentrations of α–ketoisocaproate, glutamate, glutamine and tyrosine during the insulin infusion (Table 6.2).

6.4.4 Leg Amino Acid Balance

The net leg balance of phenylalanine was just positive during the low insulin period and became markedly positive during insulin infusion, indicating that leg protein synthesis exceeded protein breakdown (Table 6.3). An increased net efflux from the leg of the gluconeogenic precursor amino acids alanine and glutamine, and also of α–ketoisocaproate, occurred during the combined insulin/amino acid infusion.

6.4.5 Enrichments of Phenylalanine, Leucine and Leucine Metabolites

Plateaux were attained for the enrichments of plasma phenylalanine, leucine and α–ketoisocaproate (Figure 6.2) and both whole blood, and expired $^{13}CO_2$ during the final periods of each phase of study. The ^{13}C enrichment of expired CO_2 was 0.0195±0.0014 APE during the initial period and 0.0271±0.0024 APE during insulin infusion. The mean value in the three subjects who did not receive tracer was

Table 6.3. Leg Net Balance of Amino Acids and α-Ketoisocaproate; Effects of Insulin During Continuous Amino Acid Infusion

	Amino Acids Alone	Insulin plus Amino Acids
	(nmol•100 g^{-1}•min^{-1})	
Alanine	-10.1±23.8	-21.8±92.6
Glutamate	120±12	146±21
Glutamine	-191±61	-343±43[a]
α-Ketoisocaproate	-2.1±3.9	-16.0±9.4
Isoleucine	27±8	102±18[b]
Leucine	24±14	143±21[b]
Phenylalanine	2.6±6.1	33.1±5.8[c]
Tyrosine	0.9±8.9	12.1±5.3
Valine	54±15	146±16[b]
BCAA	104±21	391±47[b]
Total	165±157	1139±238[b]

Statistical significance: [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.

BCAA, branched chain amino acids.

0.0008±0.0004 APE during insulin, glucose and amino acid infusion suggesting that

whole-body leucine oxidation may have been overestimated only by about 3% during this period. The leg production rate of $^{13}CO_2$ was 0.72±0.19 nmol•100 g^{-1}•min^{-1} during the initial period, and 2.58±0.40 nmol•100 g^{-1}•min^{-1} during insulin infusion; however, in the three subjects who did not receive tracer it was only -0.02±0.09 nmol•100 g^{-1}•min^{-1}, suggesting that no substantial systematic error occurred in the estimates of leg leucine oxidation due to changes in substrate metabolism by the leg.

6.4.6 Leg Protein Turnover and Amino Acid Metabolism

Leg protein breakdown, assessed with [1-^{13}C]leucine (Table 6.4) was decreased by 30% by infusion of insulin with amino acids, but an average reduction of only 9% (non-significant) could be observed using tracer phenylalanine exchange as an index. However, leg protein synthesis assessed with tracer phenylalanine (Figure 6.3) showed an increase of 55% during infusion of insulin (P=0.022), yet assessment with leucine tracer showed leg protein synthesis was, on average, only 13% greater during insulin infusion (non-significant). Oxidation of leucine by the leg increased over four fold (P=0.0029) with insulin i.e. approximately in proportion to the increase in supply of leucine. The balance between protein synthesis and protein breakdown assessed with both leucine and phenylalanine became markedly more positive during infusion of insulin, although the increase assessed by tracer leucine showed the smaller change. For entry to leg protein, the molar ratio of leucine to phenylalanine was 2.7 when the subjects were studied during low insulin conditions and was 2.0 when they were infused with insulin; in skeletal muscle protein this ratio is 2.4 (Gelfand and Barrett, 1987).

6.4.7 Whole-Body Leucine and Phenylalanine Kinetics

Whole-body leucine flux increased by 35% during the period of insulin-plus-amino acid infusion; whole-body phenylalanine flux increased by 47%. Assessment of whole-body protein breakdown with leucine tracer showed a decrease (P=0.004) of 16% during the insulin infusion; assessed with phenylalanine tracer the decrease (P=0.013) was 29%. The molar ratio of leucine to phenylalanine release from protein was 2.6 in low insulin conditions and 3.1 during high insulin-plus-amino acids. Whole-body oxidation of leucine doubled and non-oxidative leucine disappearance, i.e. whole-body protein synthesis increased (P=0.005) by 11% during the high insulin phase. The balance between protein synthesis and protein breakdown in the whole-body, assessed with leucine, changed from negative to positive (P<0.001).

6.4.8 Calculated Leg Muscle Fractional Protein Synthetic Rate

During the low insulin period, the muscle protein synthetic rate was 0.086±0.021 %/h, calculated from phenylalanine exchange, and 0.095±0.020 %/h calculated from leucine exchange. The corresponding rates during insulin infusion were 0.137±0.018 %/h and 0.110±0.020 %/h.

6.5 Discussion

The present results using either phenylalanine or leucine tracer suggest that whole-body protein breakdown is less during infusion of insulin combined with amino acids than during infusion of a lesser dose of amino acids alone, results

consistent with these of other workers (Castellino *et al*, 1987; Tessari *et al*, 1987). After insulin plus extra amino acid infusion, a modest increase was found in the rate of whole-body protein synthesis, measured by leucine turnover. A net anabolic effect on leg protein balance was indicated by results obtained with both tracers used but there were apparent discrepancies between them regarding components of the net anabolic effect: for the phenylalanine based results, a marked increase occurred in muscle protein synthesis with smaller effects on protein breakdown but for the leucine based results there was only a minor increase in the rate of muscle protein synthesis, although a marked reduction occurred in the rate of muscle protein breakdown. It is unlikely that these results have arisen purely as artifacts of the investigative and analytical techniques employed as similar discrepancies between phenylalanine- and leucine-derived limb protein turnover during combined insulin plus amino acid infusion have been reported in preliminary form by other workers who employed radiolabelled phenylalanine and leucine to assess protein turnover (Luzi *et al*, 1989). They reported that a combined insulin and amino acid infusion caused a marked increase in phenylalanine uptake but only a minimal increased in leucine uptake; the extent of the apparent discrepancy they observed between the results using the two different tracer amino acids would have been greater if leucine metabolism (ie transamination and oxidation) had been stimulated by exogenous amino acid provision, as in the present study. It is, of course, possible that our methods obscured the true extent of the decrease in muscle protein breakdown with phenylalanine and likewise of the increase in protein synthesis with leucine, causing type 1 errors, but, even if this were the case, there remains a substantial discrepancy in the apparent magnitude of the

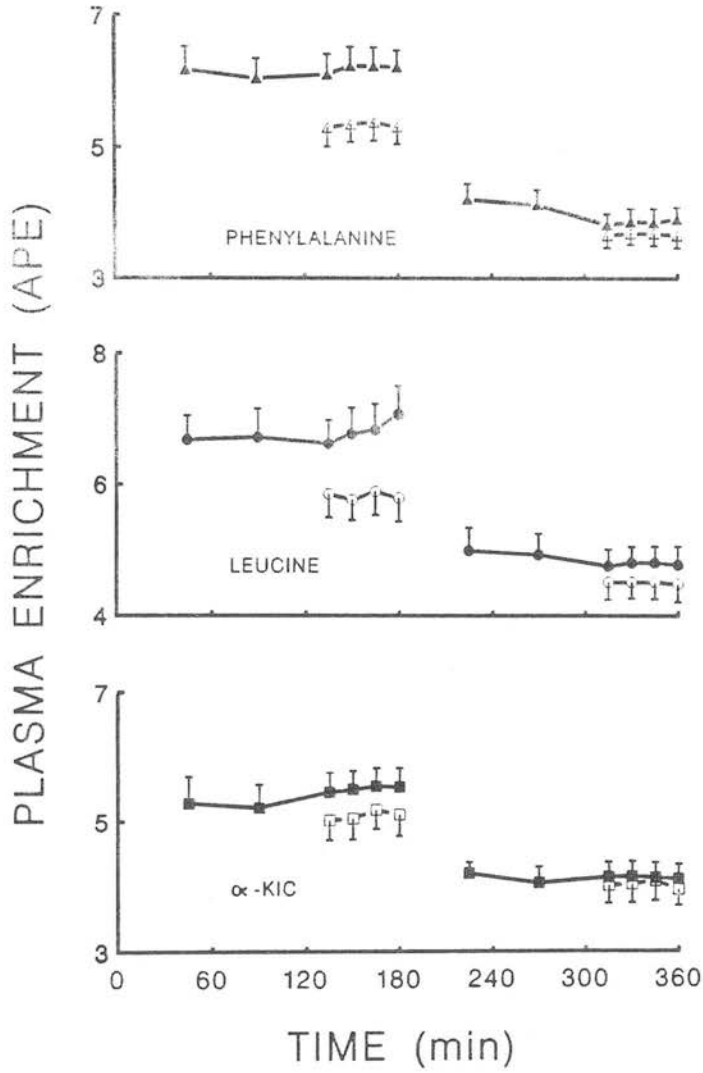

Figure 6.2. Plasma phenylalanine, leucine and α–ketoisocaproate enrichments before and during insulin infusion with continuous amino acid infusion (arterial, solid symbols; venous, hollow symbols)

changes in protein turnover induced by insulin with amino acids when assessed by leucine and phenylalanine tracers.

Before attempting to interpret the present results, whether concordant or discrepant, in physiological terms, some of the uncertainties associated with them need to be identified, their sizes estimated and their possible effects on the derived data recognised. The points to be considered are (a) the attainment or not, at metabolically active sites, of plateau conditions for the tracers of interest during each of the two periods of investigation, (b) the extent to which measurements of tracer concentrations and enrichment in accessible compartments represented those at the sites of metabolism within tissues, and (c) the relative robustness of the results obtained in allowing the construction of a qualitatively accurate account of intracellular events. The principles of using tracer labelled amino acids to investigate human amino acid and protein metabolism *in vivo* and of using the enrichment of α-ketoisocaproate as a measure of leucine labelling in the metabolically active compartment of the body, have been extensively discussed by others (Matthews *et al*, 1982; Horber *et al*, 1989; Layman and Wolfe, 1987; Schwenk *et al*, 1985a) and in Chapters 2 and 4. We have employed both primary pool stochastic models with venous sampling used to define precursor pool enrichment (for both whole-body phenylalanine metabolism and leg exchange and metabolism of phenylalanine and leucine) and a reciprocal pool model with arterial α-ketoisocaproate sampling for whole-body leucine metabolism. Accordingly, it needs to be considered whether or not the routine assumptions used were made less tenuous by the experimental circumstances of our study.

The infusion of insulin, glucose and additional amino acids in the second phase of the study resulted in marked changes in the plasma concentrations of the two tracee amino acids and α-ketoisocaproate. Phenylalanine concentration

Table 6.4. Leg Phenylalanine and Leucine Metabolism; Effects of Insulin During Amino Acid (AA) Infusion

	Phenylalanine metabolism		Leucine metabolism	
	AA Alone	Insulin + AA	AA Alone	Insulin + AA
	(nmol•100 g^{-1}•min^{-1})		(nmol•100 g^{-1}•min^{-1})	
Breakdown	44.7±8.1	40.0±7.9	120±17	84±10[d]
Synthesis	47.3±11.5	73.1±7.3[a]	129±26	146±21
Oxidation	--	--	14±4	65±10[b]
Balance (Synthesis-Breakdown)	2.6±6.1	33.1±5.8[c]	8±15	62±15[a]

Statistical significance: [a]$P<0.01$, [b]$P<0.001$, [c]$P<0.05$, [d]$P<0.02$.

showed an initial rise, but both concentration and enrichment of phenylalanine had stabilized by the end of the period from which the measurements were taken for calculations of whole-body and leg flux; however, in the case of leucine an initial rise was followed by a continuous slow (3% per h) decline in concentration during insulin infusion. Nevertheless, the calculated changes for whole-body protein breakdown are qualitatively similar with the two tracers, suggesting that even if a new steady state had not been attained for leucine, it was sufficiently close to

fulfil the basic requirement for the use of the stochastic whole-body turnover model. It is, however, possible that although the plasma amino acid concentrations and labelling were in a quasi-steady state their intracellular counterparts might have still been changing at the time of sampling. If this were the case the increased uptake of amino acids apparent during insulin infusion might have represented an expansion of the intracellular pool rather than incorporation of amino acid into protein which, accordingly, would have been overestimated. Some evidence in favour of this comes from the observation of the increased net uptake of both phenylalanine and leucine by leg tissues during hyperinsulinaemia. Such uptake could be interpreted as showing the existence of a concentration gradient from plasma to some part of the intracellular fluid, and indeed we have evidence of such a gradient from studies we carried out with infusion of insulin plus amino acids in type 1 diabetic patients (Chapter 7). In those subjects the intramuscular concentrations of both leucine and total amino acids, determined by biopsy, were slightly lower during the insulin infusion period than during infusion of amino acids alone, despite marked increases in the plasma amino acid concentrations (Chapter 7). Thus a concentration gradient *did* exist, but there was no evidence of expansion of the intracellular pool at the time of sampling. If normal subjects show similar responses then the chance of pool size changes artefactually elevating calculated rates of protein synthesis becomes minimal, although changes in breakdown could be underestimated.

The reliability of the results for whole-body protein synthesis crucially depends on the measurements of leucine oxidation, and on the assumption that the precursor pool for each process is similar. If the enrichment of the leucine

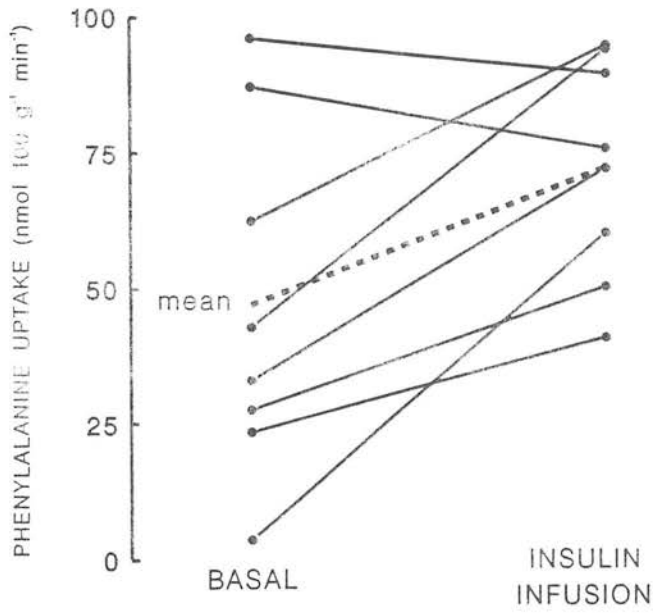

Figure 6.3. Leg muscle protein synthesis assessed by phenylalanine exchange; effect of insulin during continuous amino acid infusion

entering protein synthesis is greater than that entering leucine oxidation then synthesis will be over-estimated, and *vice versa*. There are suggestions from *in vitro* studies of rat muscle that the precursor pool tracer specific activity (ie of tRNA) for synthesis is near to that of the extracellular pool, is higher than the intracellular free pool (Section 2.3.1) and is unaffected by insulin (Airhart *et al*, 1982; Stirewalt *et al*, 1985). Recent studies in pigs (Watt *et al*, 1989) suggest that the provision of exogenous amino acids by infusion brings the tRNA labelling in muscle from a value intermediate between the extra and intracellular free pools, to a value closer to the extracellular value; the best (most easily sampled) approximation to it in all cases remains the plasma α-ketoisocaproate labelling

(Watt P.W and Rennie M.J., unpublished results). Thus, if protein breakdown is inhibited by insulin, and the rate of exchange of label among precursor pools is increased by amino acid infusion, then there remains a theoretical possibility that the apparent increases in protein synthesis after insulin and additional amino acids could have been the result of a closer approximation to the true value than could be observed at a lower rate of amino acid infusion without insulin. It seems likely that this did occur to some extent in the studies described here, possibly more so for phenylalanine where a marked increase in the plasma concentration occurred than for leucine. If this had occurred it would resulted in overestimates of the effect of insulin on protein synthesis when assessed by phenylalanine but not with leucine, but at the present time we cannot judge to what effect such an effect may have affected the results.

We have attempted to minimise the influence of the latter problem in calculating the whole-body rates by using the labelling of α–ketoisocaproate to estimate precursor enrichment. There is however a possibility that even these calculations underestimate protein synthesis in lean tissue because of a change of the site of leucine oxidation, from skeletal muscle, under low insulin conditions to adipose tissue under high insulin conditions (Frick and Goodman, 1980). Within adipose tissue the intracellular leucine pool is probably so small that almost all leucine undergoing decarboxylation would derive from plasma and therefore have a relatively high enrichment, possibly higher than that of α–ketoisocaproate; thus any tendency of the leucine metabolic model to produce overestimates of leucine oxidation should occur to a *greater* extent during infusion of insulin which stimulates decarboxylation of α–ketoisocaproate in adipose tissue (Frick and

Goodman, 1980). This of course is not simply a problem affecting whole-body rates but also the rates of synthesis in the leg, in which there are certainly metabolically active interfascicular fat depots. Accordingly, assessments of leg protein turnover with leucine might fail to reflect true increases in protein synthesis which occurred with insulin, because of diversion of leucine to oxidation in intercalated adipose tissue of the leg.

Are there any specific problems affecting the results obtained for leg metabolism? The basis of the leg metabolism calculations is different from that of the whole-body, and with consequent differences in the methodology and assumptions underlying use of phenylalanine and leucine tracers. For the leucine-based results, the equations used to assess protein synthesis depend on a greater number of analyses than for phenylalanine. These analyses include measurements of arterial and venous concentrations and enrichments not only of an amino acid, as for phenylalanine, but also of the metabolites α-ketoisocaproate and CO_2. Thus analytic variation could have a greater influence on the leucine than the phenylalanine based leg protein synthesis results. In addition, in modelling leg protein turnover with leucine a greater number of assumptions are employed than for phenylalanine with a correspondingly greater possibility of introducing systematic errors. For example, leg leucine oxidation is calculated from the enrichment of venous α-ketoisocaproate but leg protein turnover from the combined enrichment of venous α-ketoisocaproate and leucine, i.e. predominantly represented by leucine. However, except for the possibilities of *underestimating* both protein synthesis and breakdown to a lesser extent due to increased tracee concentration (particularly for phenylalanine) and *overestimating* leucine oxidation in lean leg

Table 6.5. Whole-Body Leucine and Phenylalanine Kinetics; Effects of Insulin During Amino Acid (AA) Infusion

	Leucine metabolism		Phenylalanine metabolism	
	AA Alone	Insulin + AA	AA Alone	Insulin + AA
	($\mu mol \cdot kg^{-1} \cdot h^{-1}$)		($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	
Flux	142±5	192±6[c]	60.0±2.3	88.7±4.7[c]
Infusion	25.0	93.0	15.0	56.5
Breakdown	118±5	99±6[b]	45.5±2.3	32.2±4.7[a]
Oxidation	28±2	66±3[c]	--	--
Synthesis	114±4	126±4[b]	--	--
Balance (Synthesis-Breakdown)	-4±2	26±3[c]	--	--

Statistical significance: [a]$P<0.02$, [b]$P<0.01$, [c]$P<0.001$.

All comparisons are effects due to insulin.

tissue, by falsely attributing to it rates more properly occurring in adipose tissue, it seems unlikely that the increased availability of insulin and amino acids would have added any further errors. These arguments suggest to us that the leg

metabolic model should provide at least qualitative indications of the experimental perturbations.

The main feature of the results which is internally inconsistent, and difficult for us to explain, is the lack of any apparent effect of insulin in causing a fall in breakdown of leg protein as indicated by phenylalanine tracer exchange; whole-body protein breakdown, measured by both leucine and phenylalanine, and leg protein breakdown measured by leucine tracer exchange, all appeared to fall with insulin infusion. The finding is also at variance with the results of previous workers (Gelfand and Barrett, 1987; Luzi *et al*, 1989). This may have occurred due to the rise in amino acid concentration leading to less underestimation of protein breakdown. It should be born in mind however that insulin does no appear to decrease myofibrillar protein breakdown (Smith and Sugden, 1986; Kettelhut *et al*, 1988).

Phenylalanine catabolism (hepatic hydroxylation) does not occur in muscle or fat, so the problems associated with the possible sites of leucine oxidation do not apply. However, one possibly important factor could be the large rise in availability in blood of the branched-chain amino acids which might influence the exchange of tracer phenylalanine by membrane transport effects, possibly including both *cis* and *trans* effects (Stein, 1986). In addition to competitive *cis*-inhibition of phenylalanine entry, with resultant increases in leucine entry, there may have been *trans*-stimulation of phenylalanine efflux, due simply due to the carrier moving more rapidly across the membrane when loaded than empty, and thus increasing the chances of phenylalanine being picked up for exit (Christensen and Kilberg, 1987). Such an effect might explain the apparent lack of inhibition of insulin on

leg protein breakdown, measured with tracer phenylalanine in the presence of exogenous amino acids.

As skeletal muscle protein turnover should show a single physiological response to the perturbations of amino acid availability, which of the two sets of tracer-derived results most closely reflect protein turnover? The greater increases in phenylalanine than leucine concentration will have caused artefactual *overestimation* of phenylalanine tracer-derived protein turnover values (both synthesis and breakdown), but the error in estimating the true precursor for leucine will have caused *underestimation* of the insulin associated increase in protein synthesis for the leucine based results. Furthermore the compound nature of the final leucine based estimate may have caused a Type 2 error in concluding that the leucine based synthesis did not rise. Accordingly it seems that the true extent of the effects of the insulin plus amino acids on leg synthesis lies somewhere between the two observed changes, i.e. a 15-30% increase. For reasons connected with transport phenomena the effects of insulin plus amino acids on breakdown are probably most accurately reflected by the leucine based results, i.e. a fall occurred.

Thus, after dealing with all the methodological considerations it still seems reasonable to suggest that the present results will bear interpretation in the light of the major differential features between the two phases of the study. These were that in the initial phase of the study, insulin was at low (postabsorptive) concentrations concurrent with an elevation of plasma amino acids about 38% above normal postabsorptive values, and during the second phase, plasma insulin increased due to the exogenous insulin infusion to high physiological concentrations

concurrent with a further increase of plasma amino acids to about double the postabsorptive concentration, i.e. of the same order as the maximal insulin and amino acid concentration values observed after a large mixed meal. The arterial plasma concentration of most indispensable amino acids was increased but for the femoral venous concentrations a less marked increase occurred, i.e. there was an increased amino acid balance greater than the increase in arterial supply.

What are the likely mechanisms of the anabolic effect? Analysis of the exchange of both amino acid tracers suggested that skeletal muscle protein synthesis increased, although the increase was greater on the basis of the phenylalanine tracer exchange. Skeletal muscle protein breakdown decreased significantly on the basis of leucine leg exchange. Furthermore, the same general pattern was seen for the whole-body, this consonance reinforcing the likelihood of the presumed effects and providing yet more evidence of the substantial contribution of muscle to whole-body protein metabolism.

So far as we know these observations are the first to provide evidence that under favourable conditions, insulin has a stimulatory effect on skeletal muscle and whole-body protein synthesis in man. They are thus in agreement with the results of a substantial amount of work in whole animals, in perfused organs and in isolated tissues and extracts from them (Kimball and Jefferson, 1988). The results may also hold the key to the puzzle as to why a stimulatory effect of insulin on protein synthesis, either in muscle or the whole-body, has been so difficult to observe in studies of normal and diabetic subjects previously. It now seems that (a) although insulin may, at all values of blood (and probably muscle) free amino acid concentration inhibit protein breakdown, as shown here by the leucine results

and also by previous studies of regional and whole-body metabolism (Castellino *et al*, 1987; Gelfand and Barrett, 1987; Tessari *et al*, 1987), and that (b) amino acids alone stimulate protein synthesis (Chapters 4 and 5), (c) stimulation by insulin requires a concurrently high amino acid availability, sufficient to maintain their blood and intracellular concentrations at high physiological values. We have previously shown by muscle biopsy studies in type 1 diabetic patients (Chapter 7) that modest extra provision of exogenous amino acids during insulin infusion is insufficient to prevent a fall in the intramuscular concentration of amino acids and that for some amino acids (e.g. tyrosine and methionine) insulin causes their fall to undetectable values. Even in the present study, there was evidence of a slow drift downwards of plasma amino acids during the insulin infusion despite a high constant rate of amino acid provision; for some amino acids (e.g. tyrosine, cysteine) the fall was to values below those observed before the study commenced.

It could be argued that the present results do not actually demonstrate an effect of insulin, but simply reflection a further elevation of the powerful anabolic drive provided by elevated blood amino acids. The arguments for and against this cannot yet be more than indirect, because of the current lack of definitive information on dose-responses of amino acids, or for that matter insulin, on protein synthesis in man. Nevertheless the following evidence seems to us to indicate an additional effect of insulin in the presence of high amino acid concentrations. First, a decrease occurred in the intramuscular concentration of leucine in type 1 diabetics studied during conditions equivalent to these reported here, despite similar increases in plasma leucine in the two studies (Chapter 7). Secondly, the stimulatory effect of amino acids on muscle and whole-body protein synthesis is,

in our hands, independent of the delivery of amino acids between rates of 44-88 $mg \bullet kg^{-1} \bullet h^{-1}$, as shown by the results obtained from the early phase of this study and of our previous results in normal man (Chapters 4 and 5); this point is reinforced by the reported similarity of the extent of stimulation of whole-body synthesis by a range of delivery rates of amino acids in normal man, with low physiological insulin availability (Castellino *et al*, 1987; Tessari *et al*, 1987). Thirdly, the effect is in contrast to the marked dependence of leucine oxidation on its delivery, shown here and elsewhere (Castellino *et al*, 1987; Tessari *et al*, 1987; Chapter 5), and suggests that the mechanism of the stimulation of synthesis is a non-linear function of amino acid availability, such as might occur by a covalent modification of initiation or translation factors of the synthetic apparatus (Pain, 1986; Kimball and Jefferson, 1988). Lastly, a fall in plasma tyrosine occurred, despite an increase of 3.8 fold in tyrosine supply with only a 15% decrease in the likely rate of release from protein breakdown.

Although the balance of the evidence available from the present work strongly suggests that in the presence of sufficient amino acids insulin stimulates protein synthesis in muscle and the whole-body, the present findings cannot be considered conclusive; further studies designed to confirm the results and allay our worries concerning pool sizes, transport inhibition, differential sites of oxidation, and the reasons for the different behaviour of leucine and phenylalanine tracers, and transport inhibition will need to be carried out before definitive statements can be made on the mechanisms of the net protein anabolic effects of insulin in man.

Chapter 7. INABILITY TO STIMULATE SKELETAL MUSCLE OR WHOLE-BODY PROTEIN SYNTHESIS IN TYPE 1 DIABETIC PATIENTS BY INSULIN-PLUS-GLUCOSE DURING AMINO ACID INFUSION: STUDIES OF INCORPORATION OF [1-^{13}C]LEUCINE

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FIGURES

7.1 Summary

Despite its anabolic effects on protein balance, acute administration of insulin has been reported to have no effect on skeletal muscle or whole-body protein synthesis in man. However, insulin also reduces plasma and intramuscular amino acid availability, which may limit protein synthesis. We have therefore measured the acute effects of insulin on skeletal muscle (anterior tibialis) protein synthesis and whole-body leucine turnover in eight insulin-withdrawn Type 1 (insulin dependent) diabetic patients. They were studied initially when insulin deficient, but during infusion of mixed amino acids at a rate sufficient to raise plasma amino acids by 30% ie to 4 mmol/l in total; measurements were continued when insulin was infused together with an increased rate of amino acids to maintain insulinopenic plasma amino acid concentrations. Using α-keto[1-^{13}C]isocaproate in plasma as an index of the intracellular precursor labelling, incorporation of [1-^{13}C]leucine into skeletal muscle protein was 0.068±0.007 %/h during insulin withdrawal and was unaltered during insulin infusion. Also calculated on the basis of α-ketoisocaproate labelling, non-oxidized whole-body leucine disappearance (ie whole-body protein synthesis) was 110±4 μmol•kg^{-1}•h^{-1} during insulin withdrawal; this also was unchanged during insulin infusion. Despite stable or increased plasma concentrations of most amino acids, the intramuscular concentrations of a number of amino acids decreased during insulin infusion. This may have limited any anabolic effect of insulin on protein synthesis. Alternatively, pre-existing high intramuscular amino acids may have maximally stimulated muscle protein synthesis, so that the further elevation was obscured, especially with the tendency to depletion of precursor amino acids.

7.2 Introduction

Insulin has a protein anabolic effect in mammals. Insulin deficiency in immature streptozotocin-diabetic rats depresses skeletal muscle protein synthesis, both *in vitro* and *in vivo*, which is corrected by replacement of insulin (Section 1.8.1). However, insulin stimulation of protein synthesis is difficult to reproduce in animals not rendered insulin deficient (McNurlan and Garlick, 1989). Muscle protein synthesis in postabsorptive insulin-deficient Type 1 diabetic patients is reported as neither below that in healthy subjects nor responsive to insulin (Pacy *et al*, 1989). Furthermore, although close arterial infusion of insulin (which caused no systemic fall in plasma amino acids) increased the net protein balance of the forearm in postabsorptive healthy men (Gelfand and Barrett, 1987), local protein synthesis was unaffected; the anabolism appeared to be entirely the effect of insulin in reducing forearm protein breakdown.

Studies in man suggest that the availability of amino acids, which is reduced by insulin in plasma (Castellino *et al*, 1987; Tessari *et al*, 1987; Alvestrand *et al*, 1988) and in muscle (Alvestrand *et al*, 1988), is a major factor modulating rates of whole-body (Castellino *et al*, 1987; Tessari *et al*, 1987; Chapters 4 and 5) and muscle (Chapters 4 and 5) protein synthesis. Other factors which reduce (eg 3-hydroxybutyrate (Nair *et al*, 1988b)) or increase (eg glucagon (Nair *et al*, 1987b; Almdal and Vilstrup, 1988)) amino acid oxidation may indirectly regulate protein synthesis by changing amino acid availability. Infusion of insulin plus glucose, together with amino acids sufficient to maintain plasma concentrations, had no effect on whole-body protein synthesis but appeared to reduce whole-body protein

breakdown (Castellino *et al*, 1987; Tessari *et al*, 1987) thereby improving protein balance. These results raise doubts about any physiologically important role in man for insulin in controlling whole-body protein synthesis. Nevertheless hyperaminoacidaemia during hyperinsulinaemia was accompanied by a rise in whole-body protein synthesis (Tessari *et al*, 1987; Chapter 6) and muscle protein synthesis (Chapter 6).

These results collectively suggest that any effect of insulin on protein synthesis might only be demonstrable when insulin-induced muscle amino acid deficiency, by inhibition of protein breakdown, did not constrain muscle protein synthesis. The present work was undertaken to investigate the acute effects of insulin infusion on skeletal muscle protein synthesis, determined directly with the biopsy technique, in type 1 diabetic subjects. The constraint on protein synthesis from insufficient amino acid availability was minimized by continuous infusion of a proprietary mixed amino acid solution at a rate which was increased during the insulin administration.

7.3 Methods

7.3.1 Subjects

Eight Type 1 diabetic male patients, in good health and free of major diabetic complications despite diabetes for 14±3 years, were studied. Biochemical tests indicated good diabetic control (HBA1 8.6±0.7%, normal <8.5%) and normal renal, hepatic and thyroid function. Five were completely, and three partially, deficient in C-peptide (0.13, 0.21 and 0.43 nmol/l during hyperglycaemia and

ketosis). Their total daily insulin was 52±6 U. Three additional age-matched subjects were studied without tracer to check for effects of the metabolic interventions on baseline $^{13}CO_2$ enrichments.

Table 7.1. Effect of Insulin on Plasma Hormone, Glucose and Blood D-(-)-3-hydroxybutyrate Concentrations

	Insulin Withdrawn 210-270 min	Insulin Infused 450-510 min
Free insulin (pmol/l)	33(14,75)	449(403,499)[b]
Glucagon (pmol/l)	53(30,95)	69(47,100)
Cortisol (nmol/l)	300(145,624)	306(170,548)
Insulin-like growth factor I (mU/ml)	560(330,970)	500(320,790)
Glucose (mmol/l)	12.3±2.0	6.2±0.9[a]
D(-)3-hydroxybutyrate (mmol/l)	0.53(0.16,1.74)	0.09(0.03-0.29)[a]

Data given as means, with 95% confidence limits in parenthesis.

Statistical significance: [a]$P<0.01$, [b]$P<0.001$.

7.3.2 Protocol

Each subject was studied over 240 min after overnight insulin withdrawal and then for a further 240 min during insulin and glucose infusion (Protocol b, Section 3.10.2, Figure 3.2). During the period prior to the study all subjects were prescribed high-fibre diets in which carbohydrate supplied over 50% and fat 30-40% of total energy. They were given half of their usual dose of short-acting insulin, with no intermediate or long-acting insulin, at 18.00 hours prior to the study day; they then fasted until the end of the study.

At 09.00 hours a cannula was inserted retrogradely into a dorsal hand vein for collection of arterialized venous blood samples (Section 3.5) and a contralateral forearm vein cannula was placed for all infusions. Priming doses of L-[1-^{13}C]leucine (6.84±0.25 μmol/kg) and sodium [^{13}C]bicarbonate (2.83±0.33 μmol/kg) were then administered (time 0 min) and a continuous infusion of L-[1-^{13}C]leucine (7.59±0.32 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) commenced via a contralateral forearm vein cannula. At the same time a priming dose of Synthamin 14 mixed amino acid solution (29 μmol leucine/kg, 52 mg amino acids/kg) was given over 10 min (Section 3.3, Table 3.1); Synthamin 14 was then continuously infused at a rate providing leucine 25 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ until the end of the period of insulin withdrawal (time 270 min). A priming dose (Section 3.9.2) of Humulin S neutral human insulin (Section 3.3) was then given followed by a continuous infusion at 0.29 $nmol \cdot m^{-2} \cdot min^{-1}$ (40 $mU \cdot m^{-2} \cdot min^{-1}$) and the rate of Synthamin 14 was increased to provide leucine at 93 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ until time 510 min. Glucose was infused when the plasma glucose concentration fell to 5 mmol/l.

Table 7.2. Plasma Amino Acid Concentrations Before and During Amino Acid (AA) Infusion, During Insulin Withdrawal and During Insulin Infusion

Amino acid	Before AA Infusion -15 min	Insulin Withdrawn 30-270 min (μmol/l)	Insulin Infused 330-510 min
Alanine	266±24	423±92[c]	791±190[d]
Arginine	133±21	203±26[d]	304±39[d]
Asparagine	44±3	47±3[b]	29±2[d]
Glutamic acid	61±8	60±25	48±23[c]
Glutamine	632±45	695±140[c]	612±94[b]
Glycine	229±23	369±40[d]	588±59[d]
Histidine	99±5	141±8[d]	200±15[d]
Isoleucine	110±15	183±19[d]	274±25[d]
Leucine	211±24	333±32[d]	407±39[c]
Lysine	202±12	257±18[c]	328±32[b]
Methionine	27±2	52±6[d]	103±12[d]
Phenylalanine	64±2	96±4[d]	161±7[d]
Serine	131±17	191±28[c]	251±34[d]
Taurine	66±6	64±5	47±3[d]
Threonine	145±18	198±28[c]	247±37*
Tryptophan	48±4	60±3	96±6[d]
Tyrosine	57±3	54±4[a]	41±3[d]
Valine	320±32	431±37[d]	588±60[c]
Total amino acids	2950±150	4000±240[d]	5120± 350[d]

Comparisons are 30-270 min *vs* -15 min and 330-510 min *vs* 30-270 min.
Statistical significance: [a]$P<0.05$, [b]$P<0.02$, [c]$P<0.01$, [d]$P<0.001$.

Thirty min after priming with [1-^{13}C]leucine and beginning the mixed amino acid infusion (ie elapsed time 30 min), a percutaneous muscle biopsy was obtained from the tibialis anterior, a second muscle biopsy was taken from the same or the contralateral leg 240 min later at the end of the insulin withdrawal period, and a third biopsy from the leg contralateral to that first biopsied at time 510 min, the end of the insulin infusion period (Section 3.7).

Blood samples were obtained before tracer administration, at the time of the first muscle biopsy (30 min), and every 60 min thereafter (Section 3.5). Coincident samples of expired air were collected for subsequent determination of $^{13}CO_2$ enrichment (Section 3.6). Total carbon dioxide production and oxygen consumption were determined for 11 min every 60 min with a ventilated-hood indirect calorimeter (Section 3.6).

In all subjects we avoided interference with the estimation of [1-^{13}C]leucine oxidation from oxidation of maize-derived dextrose, which has a high natural abundance of ^{13}C, by the use of potato starch glucose, which does not (Section 3.3). In the three control subjects who did not receive ^{13}C tracer, breath samples were obtained to measure possible background changes, including the effects of increased amino acid oxidation, in the ^{13}C enrichment of expired CO_2.

An automated amino acid analyzer with fluorometric detection (Section 3.13) was used to measure concentrations of amino acids from plasma and intramuscular water, the latter calculated on the assumption that 13% of the muscle biopsy water was extracellular (Bergström *et al*, 1974).

Plasma α-ketoisocaproate ^{13}C enrichment was used to calculate whole-body leucine rates of appearance (Ra) and of disappearance (Rd) with non-steady state equations (Section 2.3.2).

Mean and SEM values are presented; for D-(-)-3-hydroxybutyrate and hormones, which showed log-normal distributions, the values were transformed to their natural logarithms before analysis; the means and 95% confidence intervals presented are the antilog$_e$ transformations.

7.4 Results

7.4.1 Indirect calorimetry

During insulin withdrawal carbon dioxide production was 204±7 ml/min and increased by 11% during insulin infusion ($P<0.01$); respiratory quotient was 0.72±0.01 and increased to 0.75±0.01 ($P<0.01$); calculated heat production was 5.69±0.26 kJ/min and increased by 5% to 6.00±0.26 kJ/min during insulin replacement ($P<0.01$).

7.4.2 Hormones and Substrates

Blood bicarbonate before infusion was 23.1±0.5 μmol/l and remained in the range 22-25 throughout. Plasma hormone concentrations were stable within each phase of the study (Table 7.1. During insulin infusion, plasma free insulin concentration increased from low (33 pmol/l) to high physiological values (449 pmol/l). Plasma glucagon, cortisol and insulin-like growth factor 1 were unchanged. Plasma glucose and blood D-(-)-3-hydroxybutyrate were stable during

Table 7.3. Effect of Insulin on Intramuscular Free Amino Acid Concentrations During Amino Acid Infusion

Amino acid	Insulin Withdrawn		Insulin Infused
	30 min	270 min	510 min
		(μmol/l)	
Alanine	1920±140	2090±180	3340±160[d]
Arginine	608±104	598±112	607±113
Asparagine	897±44	914±71	850±67
Glutamic acid	4750±300	4820±460	3050±210[c]
Glutamine	13400±800	13200±1900	11600±900[a]
Glycine	1510±100	1570±90	1640±110
Histidine	597±72	652±95	562±85[a]
Isoleucine	149±17	175±31	173±33
Leucine	298±32	341±9	282±50[c]
Lysine	1808±257	2214±286	1376±344
Phenylalanine	95±8	105±8	145±9[d]
Serine	690±75	670±86	650±84
Taurine	17600±900	17700±1300	16100±1100
Threonine	769±56	729±68	705±68
Tyrosine	89±6	81±4[b]	21±7[d]
Valine	448±79	444±79	492±100
Total amino acids	45200±1300	45000±2100	41200±170[a]

Comparisons are 30 min *vs* 270 min and 270 min *vs* 510 min.

Statistical significance: [a]$P<0.05$, [b]$P<0.02$, [c]$P<0.01$, [d]$P<0.001$.

insulin withdrawal and decreased during insulin infusion. During the final 60 min of insulin infusion the rate of glucose infusion was 2.17±0.64 $mg \cdot kg^{-1} \cdot min^{-1}$.

7.4.3 Amino and Keto Acid Concentrations

Mean plasma leucine concentration (Figure 7.1; Table 7.2) was 211±24 μmol/l before the start of the primed amino acid infusion. The plasma leucine concentration increased by 13% throughout the period when leucine kinetics were calculated during insulin withdrawal ($P<0.02$); mean plasma leucine was 337±33 μmol/l during this period. The plasma leucine concentration decreased by 18% throughout the period when leucine kinetics were calculated during insulin infusion ($P<0.01$); the mean concentration during this period (407±39 μmol/l) was 21% higher than during insulin withdrawal ($P<0.01$). Plasma α–ketoisocaproate concentration was 53±9 μmol/l during insulin withdrawal and was not significantly different during insulin infusion (43±7 μmol/l). The total concentration and concentrations of most individual amino acids in plasma (Table 7.2) were increased by the amino acid infusion given at a faster rate during insulin administration. Exceptions were asparagine, glutamic acid, glutamine, tyrosine (Figure 7.1) and taurine which all decreased. The concentration of free leucine in intramuscular water (Figure 7.1, Table 7.3) was not different from the plasma concentration before insulin, ie at 30 or at 270 min. During insulin infusion the intramuscular leucine concentration decreased (when sampled at the end, ie at 510 min) and was actually lower than both the plasma value at the same time ($P<0.01$) and the preceding intramuscular value ($P<0.01$). Despite the general increase in plasma amino acid concentrations when insulin plus additional amino acids were infused

a reduction was found in the concentration of total amino acids in intramuscular water. The biggest change occurred for tyrosine which decreased from 81±4 µmol/l to a mean of only 21±7 µmol/l; it was reduced below detectable values in three subjects (Figure 7.1).

7.4.4 Plasma and Muscle Free Leucine Pool Enrichments

For both plasma α-ketoisocaproate and for plasma leucine plateaux of ^{13}C enrichment were observed during insulin withdrawal and significantly reduced plateaux were found during insulin replacement (Table 7.4, Figure 7.2). However, leucine enrichment in muscle was unchanged. No differences occurred in the ratios of enrichment of free leucine and its metabolite in plasma or muscle or between them as a result of insulin administration.

7.4.5 Muscle Protein Synthesis

Mixed skeletal muscle protein synthesis (k_s) was 0.068±0.007 %/h during insulin withdrawal (Figure 7.3) and was unchanged (0.071±0.006 %/h) during insulin replacement. If muscle protein synthesis rates were calculated on the basis of the enrichment of plasma or intracellular leucine (Table 7.5), they tended to be lower or were higher than on the basis of the plasma α-ketoisocaproate labelling, respectively, but no effects of insulin-plus-glucose were seen. From the present α-ketoisocaproate based results it can be calculated that there was a 83% likelihood of detecting an increase of 30% in skeletal muscle protein synthesis during insulin infusion (α = 0.05, two-tailed test); thus the chance of a Type II error was only 17%. Individual subjects showed different changes in k_s during

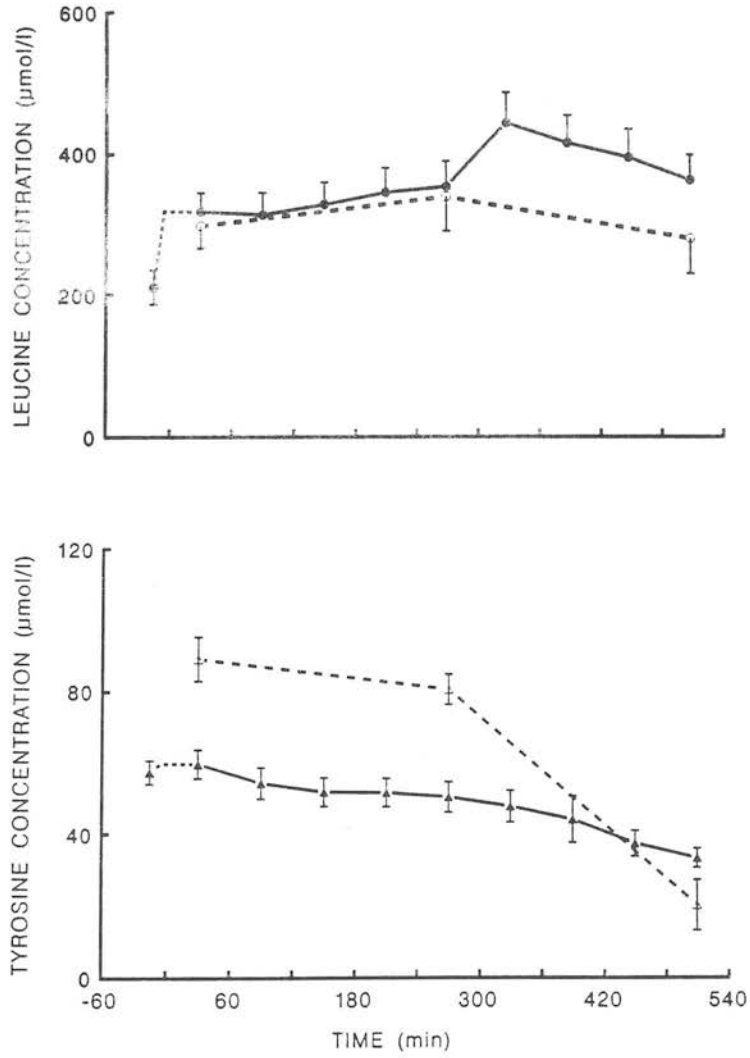

Figure 7.1. Upper panel: plasma (solid line) and intramuscular (dotted line) leucine concentrations. Lower panel: plasma and intramuscular tyrosine concentrations

insulin infusion but on stepwise regression analysis no correlation was found between the changes in k_s and changes in the concentration of

D-(-)-3-hydroxybutyrate, changes in the respiratory quotient or the absolute glucose infusion rate during hyperinsulinaemia.

7.4.6 Whole-Body Leucine Kinetics

Expired CO_2 ^{13}C enrichment was 0.0188±0.0013 atoms % Excess (APE) during insulin withdrawal and was higher during insulin infusion (0.0298±0.0012 APE, $P<0.001$). The corresponding mean values were 0.0002±0.0001 and 0.0009±0.0005 APE in three subjects not infused with ^{13}C tracer leucine, suggesting that leucine oxidation may have been overestimated by only 3% during the latter period due to $^{13}CO_2$ released from oxidation of the infused glucose.

Leucine oxidation was 35±3 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ during insulin withdrawal and increased by 84% during insulin replacement ($P<0.001$) (Table 7.6). Whole-body leucine turnover was stable during the final 180 min of each phase of the study (Figure 7.4). Total leucine appearance (Table 7.6) was 150±5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ during insulin withdrawal and increased 18% during insulin replacement ($P<0.01$); endogenous leucine appearance (protein breakdown) was 125±5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ and decreased by 33% ($P<0.01$). On the basis of plasma α–ketoisocaproate labelling, total leucine disappearance (Figure 7.4) was 145±5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ during insulin withdrawal and increased by 28% during insulin replacement ($P<0.01$); the non-oxidized component of Rd (whole-body protein synthesis) was 110±4 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ and was not significantly different during insulin replacement (117±4 $\mu mol \cdot kg^{-1} \cdot h^{-1}$). However if the plasma leucine labelling was used instead of α–ketoisocaproate and the non-steady equations were applied, then whole-body protein synthesis increased from 96.3±5.4 to 119.1±7.1 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ ($P<0.01$). On

Table 7.4. Enrichments of Plasma and Intramuscular Free Leucine and α-Ketoisocaproate, and Their Ratios, During [1-^{13}C]Leucine Infusion

	Insulin Withdrawn (90-270 min)	Insulin Infused (330-510 min)
Plasma leucine (APE)	5.01±0.21	4.19±0.18[‡]
Plasma α-ketoisocaproate (APE)	4.70±0.31	4.19±0.23[a]
Intramuscular leucine (APE)[d]	3.82±0.27[c]	3.33±0.32[c]
Plasma α-ketoisocaproate/plasma leucine	0.94±0.05	0.99±0.03
Intramuscular leucine/plasma leucine	0.76±0.04	0.80±0.07
Intramuscular leucine/plasma α-ketoisocaproate	0.82±0.05	0.80±0.06

Comparisons are 330-510 *vs* 270-90 min. Statistical significance: [a]$P<0.02$, [b]$P<0.001$. [c]Intramuscular leucine enrichment was significantly different ($P<0.02$) from plasma leucine and plasma α-ketoisocaproate at 90-270 and 330-510 min. [d]Intramuscular leucine was measured at 270 min and 510 min.

this basis there was also a reduction in whole-body protein breakdown which was highly significant ($P<0.001$) but it was only by 23% of the pre-insulin value (results not shown).

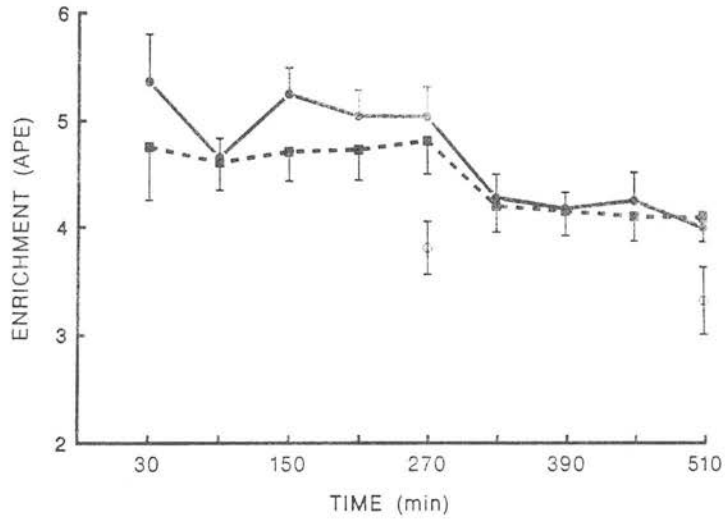

Figure 7.2. Plasma leucine (solid line) and α-ketoisocaproate (broken line), and intramuscular free leucine (hollow circles) ^{13}C enrichments

7.5 Discussion

In the investigations described here plasma free insulin was increased by insulin infusion from low to high physiological concentrations over four hours. During insulin infusion, increased infusion of amino acids resulted in indispensable amino acids being maintained at, or slightly above, plasma concentrations seen before insulin. Despite the increased supply of amino acids no significant increase occurred in skeletal muscle protein synthesis during insulin replacement, irrespective of the precursor used as the basis of the calculations.

A number of possibilities might explain this negative result. Although it is possible that in the adult human body the capacity for modulation of muscle

protein synthesis by insulin is absent, this seems unlikely, especially in view of the extensive evidence from animal studies (Section 1.8.1). Alternatively, insulin may have been ineffective on account of a post-receptor defect of protein metabolism in the diabetic patients (whose glucose metabolism was markedly insulin resistant, as indicated by the low respiratory quotients and glucose disposal rates), but the possible nature of such an effect is presently unknown.

That the rates of anterior tibialis muscle protein synthesis found in the present study during insulin withdrawal tended to be faster (P=0.12) than those in 7 healthy postabsorptive subjects studied by identical means (Chapter 4) may offer a further possibility. It seems that at low concentrations of insulin muscle protein synthesis was not pathologically reduced in these diabetic subjects and may even have been elevated, a result in accordance with the results from Halliday's group (Pacy *et al*, 1989): thus skeletal muscle protein synthesis may have been already maximally stimulated in the diabetic patients and further increase by insulin may not have been possible. This would be in marked contrast to the situation in immature rats in which muscle protein synthesis is reduced at low insulin concentrations (Section 1.8.1).

There is also the slight possibility that if insulin were initially totally absent, instead of present at very low concentrations (Table 7.1), it might have been possible to observe an impairment of basal skeletal muscle protein synthesis in our patients, since in the rat at least sensitivity of protein synthesis to insulin is already maximal over 0-10 mU/l (Millward *et al*, 1983). Evidence against this comes from our findings that two subjects in whom insulin was not detectable by our assay had muscle protein synthetic rates comparable with those of the other

subjects in whom it was. Nevertheless this argument cannot be conclusive since the range of insulin sensitivity of protein synthesis in adult man is unknown and may differ markedly from that in young rats and other animals.

The possibility of limitation of intracellular amino acid availability remains to be considered. Amino acid infusion at an increased rate did not prevent an insulin-induced reduction in the muscle water concentration of certain amino acids, most markedly for tyrosine. This reduction may have acted as a constraint on muscle protein synthesis (even though the affinity of tRNA for amino acids is very high *in vitro* (Tyobeka and Manchester, 1985)) or the normal stimulatory effect (Chapter 4) of amino acids on muscle protein synthesis may have been reduced. The amino acid mixture used contained only a small quantity of tyrosine, whose relative insolubility limits its delivery by infusion. Although not indispensable in the whole-body in adults, usually being produced in liver from phenylalanine, it is essential in muscle which cannot synthesize it; the ratio between protein-bound and free tyrosine is high, so that the "safety factor" for tyrosine availability is small (Waterlow *et al*, 1978b). Decreases in the plasma tyrosine may have limited uptake of tyrosine into the limb since its net uptake depends upon the plasma concentrations (Lundholm *et al*, 1987). Its uptake, by the insulin-independent L–system transporter (Yudilevitch and Boyd, 1987; Hundal *et al*, 1989), may also have been competitively inhibited by the relatively high concentrations of the branched-chain amino acids, phenylalanine and tryptophan; furthermore these amino acids may have depleted muscle tyrosine by trans-stimulation (Stein, 1986) of the L–system.

In comparison to healthy postabsorptive subjects (Chapter 4), the patients with diabetes we studied had markedly raised plasma amino acid concentrations during insulin withdrawal, partly, presumably, as a result of increased protein breakdown as well as amino acid infusion. It seems likely that elevated amino acid availability initially may have contributed to the elevated muscle protein synthetic rates we measured in the insulin withdrawn state, since we have shown previously that muscle protein synthesis in healthy postabsorptive men increased by an average of 35% during infusion of mixed amino acids alone (Chapter 4), and we have recently confirmed the effect by arterio-venous tracer amino acid flux measurements in a separate group of subjects (Chapter 5). It is difficult to choose which of two possible effects, ie stimulation by an initially high intramuscular amino acid concentration or limitation by removal of muscle essential amino acids was most important for the lack of a further stimulation by insulin, but both could conceivably have been involved. In any event, our present and previous findings are consistent with the proposition that, in man, availability of amino acids rather than of insulin, is important in maintaining rates of skeletal muscle protein synthesis, and that insulin is less so.

A number of other substrates which could have influenced muscle protein synthesis changed in response to insulin infusion. 3-Hydroxybutyrate apparently stimulates muscle protein synthesis, but by only 10% as its concentration increases over a fourfold range from 0.5 mmol/l (Nair *et al*, 1988b): we found that insulin caused a small decrease in 3-hydroxybutyrate, making it unlikely that such a change would have relieved a previously stimulatory effect on skeletal muscle protein synthesis. In rat diaphragm and heart muscle *in vitro*, glucose increases

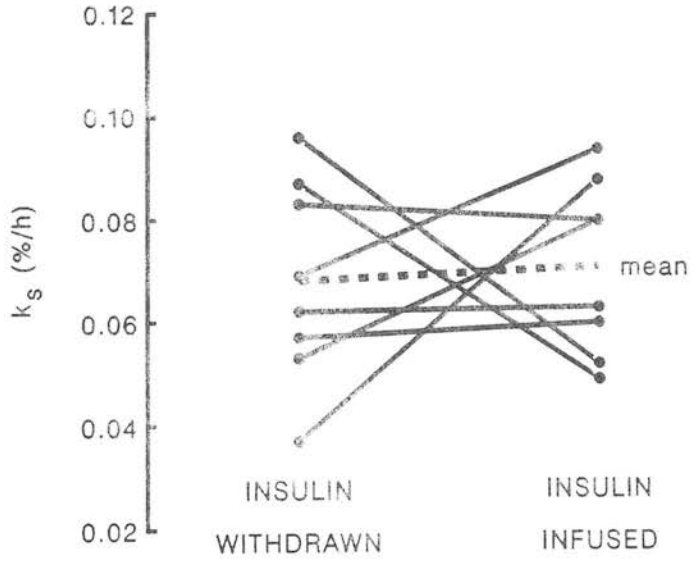

Figure 7.3. Individual and mean skeletal muscle protein synthesis rates during insulin withdrawal and replacement with continuous amino acid infusion

protein synthesis (Hedden and Buse, 1982) and deficiency of glucose results in increased leucine oxidation (Hedden and Buse, 1982). Plasma glucose was elevated during insulin withdrawal but muscle was unlikely to have been able to utilize it efficiently because of lack of insulin and little sparing of amino acid catabolism could have occurred. Other factors not otherwise considered in our patients include elevations of fatty acids and medium-chain triglycerides, the latter being reported to improve forearm balance of amino acids (Wicklmayr *et al*, 1987).

In addition a number of hormones may have altered in concentration during insulin infusion. Growth hormone (not measured) may have been initially elevated but the patients were in good diabetic control, and plasma insulin-like growth

factor I was in the normal range making this unlikely. Paracrine and systemic growth factors other than insulin-like growth factor I may influence protein synthesis in ways not accessible to our analyses. During insulin infusion plasma free insulin increased into the high physiological range but nevertheless remained beneath the threshold necessary to stimulate insulin-like growth factor receptors. However insulin-like growth factor I was measured in plasma only and any local muscle-cell changes would not have been apparent. We suggest that this is unlikely not only because of a lack of any anabolic effect, but also because increases in insulin-like growth factor I normally require considerable exposure to insulin (Glaser *et al*, 1987).

Marked changes in the rates of muscle protein synthesis were evident between patients; increases occurred in three, but in two patients there were decreases. Stepwise regression analysis showed these changes not to correlate either with indices of insulin sensitivity or changes in the plasma 3–hydroxybutyrate. Although it is not possible with the present study design to identify specific factors which induced increases in protein synthesis in particular individuals during insulin infusion, the occurrence of the increases suggests that there may have been some stimulation of protein synthesis by a combination of insulin and some unidentified factor.

The muscle biopsy technique used, although invasive, enables measurement of muscle protein synthesis with few inherent assumptions. The plasma enrichment of α–ketoisocaproate during infusion of leucine tracer is assumed to represent the labelling of leucyl-tRNA within cells: we have recently obtained good evidence that for pig muscle, with and without infusion of amino acids, it comes closer to the

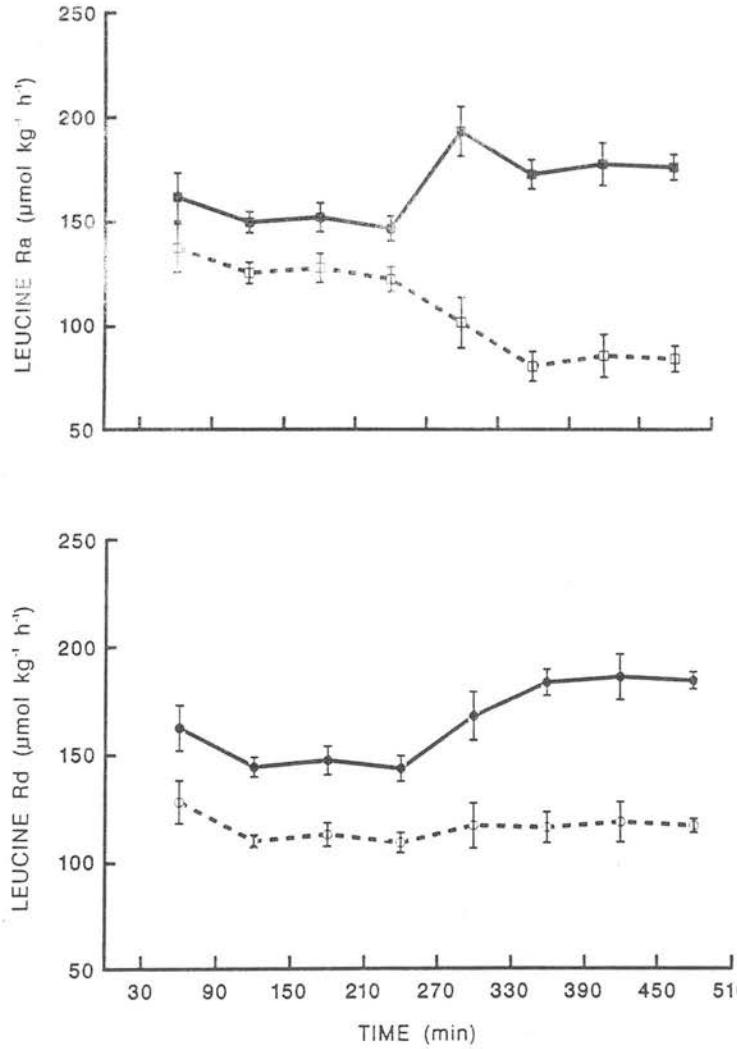

Figure 7.4. Upper panel: whole-body total leucine appearance (solid line) and endogenous leucine appearance (broken line). Lower panel: total leucine disappearance and non-oxidized leucine disappearance

leucyl-tRNA than do a number of other possible indices such as plasma or intracellular leucine (Watt *et al*, 1989). Only circumstantial evidence concerning

this question is currently available in man, but it is consistent with our approach (Matthews *et al*, 1982; Schwenk *et al*, 1985a). Given the present study conditions, in which plasma leucine concentration was high throughout both phases of investigation, tracer exchange between plasma and the intracellular fluid was likely to be rapid; certainly, we observed ratios of the enrichment of plasma α–ketoisocaproate/leucine, intramuscular leucine/plasma leucine and intramuscular leucine/α–ketoisocaproate much closer to unity in the diabetic patients than in healthy subjects studied without infusion of amino acids (Chapter 4). There is, therefore, less variation in the magnitudes of rates of muscle protein synthesis in the diabetic patients than in healthy subjects (Chapter 4) when different precursor pools are used to calculate the muscle protein synthesis rate.

The rates of whole-body protein leucine kinetics are in agreement with values published by other workers (Nair *et al*, 1983; Tessari *et al*, 1986a; Nair *et al*, 1987a). The apparent lack of stimulation by insulin of whole-body protein synthesis in the diabetic patients is consistent with results from healthy subjects showing that rates of protein synthesis correlated only with plasma leucine (Castellino *et al*, 1987; Tessari *et al*, 1987). In the present study the observed lack of insulin stimulation of skeletal muscle is consistent with a lack of change in whole-body protein synthesis. That leucine oxidation increased with insulin infusion is also in agreement with previous work in which plasma leucine was maintained during insulin infusion (Castellino *et al*, 1987). Although insulin deficiency increases leucine oxidation in perfused hind-limbs, from fasted or fed rats, its replacement reduces leucine oxidation in the fed preparation but markedly increases it in the fasted preparation (Hutson *et al*, 1980). Also, insulin has been

Table 7.5. Anterior Tibialis Muscle Protein Synthetic Rates (k_s) Calculated on the Basis of Enrichment of Various Possible Precursors

Possible Precursor	Insulin Withdrawn (k_s, %/h)	Insulin Infused (k_s, %/h)
Plasma leucine	0.063±0.007	0.071±0.007
Plasma α-ketoisocaproate	0.068±0.007	0.071±0.006
Intramuscular leucine	0.084±0.010[a]	0.091±0.007[a]

Statistical significance: [a]$P<0.05$ in comparison with plasma leucine or α-ketoisocaproate based synthetic rates.

shown to increases leucine oxidation in adipose tissue *in vitro* (Frick and Goodman, 1980). It appears likely that the increased leucine oxidation we observed during insulin infusion occurred in muscle and adipose tissue. The extent of leucine oxidation in different human tissues *in vivo* is not well documented although the distribution of the α-keto acid dehydrogenase (Khatra *et al*, 1977) suggests muscle to be a major site, and our own arterio-venous measurements suggest that it must be the major one in healthy man (Cheng *et al*, 1985; Chapter 5).

The measurement of muscle protein synthesis uses fewer uncheckable assumptions than the assessment of whole-body protein synthesis with the tracer

Table 7.6. Effect of Insulin on Whole-Body Leucine Kinetics During Amino Acid Infusion

	Insulin Withdrawal	Insulin Infusion
	($\mu mol \cdot kg^{-1} \cdot h^{-1}$)	
Total Flux	150±5	176±5[a]
Infusion	25	93
Breakdown (Endogenous Ra)	125±5	83±5[b]
Total Rd	145±5	185±4[b]
Synthesis (Non-Ox Rd)	110±4	117±4
Oxidation	35±3	68±4[b]

Statistical significance: [a]$P<0.01$, [b]$P<0.001$.

Ra = rate of appearance and Rd = rate of disappearance.

dilution technique. In addition to uncertainties of precursor pool labelling (Chapter 4), changes in insulin action result in alterations of plasma and intramuscular leucine concentration with the result that whole-body protein synthesis, calculated with steady state equations, tends to be overestimated during insulin withdrawal with the opposite effect during insulin replacement. Although not independently validated the equations that we used take account of the changing tracee

concentrations. A further source of error which may have caused underestimation of protein synthesis during the present study conditions is recycling of tracer, which may leads to increased plasma tracee enrichment and underestimation of both flux and protein synthesis despite coincident underestimation of leucine oxidation; however this is usually only a problem in more prolonged infusions (Schwenk *et al*, 1985b). In the present investigations a bicarbonate recovery value of 0.81 was used, derived from intravenous tracer-bicarbonate infusion in healthy postabsorptive subjects. As determined from the appearance of tracer-carbon labelling in glucose by incorporation of the carbons during gluconeogenesis, the proportion of bicarbonate fixed during infusions of leucine tracer from oxidation of leucine carboxyl-carbon within mitochondria is greater than occurs during infusion of bicarbonate for the same degree of plasma bicarbonate labelling. Therefore, general use of a bicarbonate recovery value of 0.81 may lead to additional underestimation of leucine oxidation and thus, overestimation of protein synthesis. During provision of glucose, which stimulates insulin secretion and inhibits gluconeogenesis, fixation of tracer-carbon from bicarbonate by gluconeogenesis is reduced (McMahon *et al*, 1989) and under these conditions the figure of 0.81 may markedly overestimate leucine oxidation exacerbating the underestimation of protein synthesis. Changes also occur in the whole-body bicarbonate pool during insulin withdrawal and insulin replacement inducing additional errors in estimates of bicarbonate fixation and leucine oxidation. We probably avoided one source of error in glucose infusion: if maize derived glucose had been infused $^{13}CO_2$ released from maize glucose oxidation would have led to an overestimation of leucine oxidation and a further corresponding underestimation of whole-body protein

synthesis during hyperinsulinaemia. Such factors collectively result in underestimation of whole-body protein synthesis by the tracer-leucine dilution technique during hyperinsulinaemic conditions and may induce qualitative errors in the interpretation of the effects of insulin action on whole-body protein synthesis, both in our work and that of others (Castellino *et al*, 1987; Tessari *et al*, 1987).

Comparison of the present whole-body leucine metabolism results with those from studies in non-diabetic men, in which where whole-body protein synthesis was not stimulated by insulin (Castellino *et al*, 1987; Tessari *et al*, 1987), are consistent with a lack of any fundamental difference in sensitivity of amino acid metabolism to insulin between diabetic and non-diabetic subjects (Tessari *et al*, 1986a). Similar conclusions might be drawn by comparing the skeletal muscle leucine incorporation results reported here for Type 1 diabetic subjects with the reported lack of effect of insulin on forearm arteriovenous uptake of phenylalanine in healthy man (Gelfand and Barrett, 1987). In contrast, our experience with the arteriovenous exchange technique in healthy man suggests that when amino acids are adequately replaced during insulin infusion there was a stimulatory effect of insulin on both whole-body and muscle protein synthesis, the latter apparent when assessed with phenylalanine but not with leucine (Chapter 6). Possible explanations include: the possibility that differences *do* exist between type 1 diabetic patients and healthy subjects (see Chapter 8), possibly due to either insulin resistance in type 1 diabetic patients or that they were studied immediately after a period of insulin withdrawal. A second possibility is that the *average* rate of skeletal muscle protein synthesis was not increased during the 4 h of insulin replacement, the period

would included a period before insulin had any effect and possibly a period when reduced amino acid availability constrained protein synthesis. A third possibility is that one or other model of protein metabolism (ie leucine incorporation assessed by biopsy and amino acid uptake assessed by amino acid exchange) may be deficient; the different methods used in the two investigations, and the differences in the subjects studied may make comparisons invalid.

To conclude, in the present study we could not show any acute anabolic effect of systemic insulin infusion on skeletal muscle protein synthesis in insulin-withdrawn adult diabetic patients. It appears possible that reduced intracellular concentrations for a number of amino acids during insulin infusion, in particular tyrosine, may have constrained muscle protein synthesis. The hypothesis that such a deficiency of amino acids, particularly tyrosine, is the reason why protein synthesis is not stimulated by insulin needs to be further investigated using amino acid mixtures including tyrosine dipeptides (Albers *et al*, 1988) to increase its availability.

In the meantime the likelihood that increased total amino acid availability maintains muscle protein synthesis in diabetic subjects and that acute treatment with insulin does not stimulate it further must weaken the perception that the generally anabolic effect of insulin includes stimulation of muscle protein synthesis. It may be however, that in normal healthy subjects or in diabetic patients with physiological amino acid concentrations, insulin does stimulate muscle protein synthesis, if sufficient substrate is supplied; the fact that Type 1 insulin dependant diabetic children may grow normally suggests that this is so.

Chapter 8. INSULIN RESISTANCE OF SKELETAL MUSCLE PROTEIN SYNTHESIS IN TYPE 1 DIABETIC PATIENTS

CONTENTS

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FIGURES

8.1 Summary

To assess whether leg and whole body protein metabolism is insulin resistant in type 1 diabetic patients this was measured with [^{15}N]phenylalanine and [1-^{13}C]leucine models. Eight type 1 diabetic patients were studied twice and eight healthy nondiabetic control subjects were studied once during hypoinsulinaemic conditions and again during infusion of insulin (0.29 nmol•m^{-2}•min^{-1}). Studies in the diabetic patients were undertaken both without amino acid infusion (-I-AA and +I-AA) and in both groups with infusion of a mixed amino acid solution (-I+AA and +I+AA). In the insulin withdrawn diabetic patients insulin alone abolished the negative leg phenylalanine balance due to a 42% reduction (P<0.05) in muscle protein breakdown and no change in muscle protein synthesis. In the amino acid infused diabetic patients insulin combined with additional amino acids increased phenylalanine-based muscle protein synthesis (-I+AA 33.1±8.0 *vs* +I+AA 45.7±7.5 nmol•100 g^{-1}•min^{-1}, P=0.077) and reduced muscle protein breakdown 41% (43.8±7.1 *vs* 25.9±3.7 nmol•100 g^{-1}•min^{-1}, P<0.05). Leg leucine oxidation was unchanged by insulin alone but was increased during insulin infusion with additional amino acids (-I+AA 10.3±2.6 *vs* +I+AA 40.8±8.5 nmol•100 g^{-1}•min^{-1}, P<0.01). In the diabetic patients insulin and amino acid stimulated phenylalanine-based leg protein synthesis (45.7±7.5 nmol•100 g^{-1}•min^{-1}) and leg glucose uptake (1.60±0.28 μmol•100 g^{-1}•min^{-1}) were impaired in comparison to the nondiabetic subjects (protein synthesis: 73.1±7.3 nmol•100 g^{-1}•min^{-1}, P=0.021; glucose 3.37±0.28 μmol•100 g^{-1}•min^{-1}, P=0.040). These results suggest that in type 1 diabetic patients: a) infusion of insulin only stimulates leg protein synthesis when combined

with a substantial provision of amino acids, and b) muscle protein synthesis exhibits impaired sensitivity to insulin, as does glucose uptake.

8.2 Introduction

Type 1 diabetic patients have both a deficiency of endogenous insulin secretion and a well documented insensitivity to insulin by glucose disposal in the whole-body (DeFronzo *et al*, 1982; Del Prato *et al*, 1983; Pernet *et al*, 1984) and specifically in muscle (Butterfield and Whichelow, 1964; Hother-Nielsen *et al*, 1987; Beck-Nielsen, 1989; Bogardus, 1989). Lipid metabolism in type 1 diabetes is also relatively insulin resistant (Jensen *et al*, 1989). In addition there is an impaired effect of insulin on whole-body protein metabolism assessed by leucine-tracer dilution techniques in type 1 diabetic patients, in comparison to nondiabetic subjects, in whom there are decreased reductions in the rates of whole-body protein breakdown and leucine oxidation during euglycaemic hyperinsulinaemia (Tessari *et al*, 1986a; Tessari *et al*, 1988). In man insulin is thought to exert a protein anabolic effect in the whole-body predominantly by reducing the rate of protein breakdown (Section 1.8.1; Chapter 6; Chapter 7). The anabolic effect in the whole-body may also include modulation of protein synthesis when amino acids are maintained at comparable concentrations by exogenous infusion (Chapter 6). Assessment of human muscle protein metabolism *in vivo* in healthy subjects using a phenylalanine-tracer-based arteriovenous exchange technique suggests that insulin reduces the rate of protein breakdown (Gelfand and Barrett, 1987) but does not alter protein synthesis. However, when amino acid availability is increased by amino acid infusion, insulin predominantly exerts its anabolic effect by increasing

protein synthesis (Chapter 6). In contrast direct assessment of muscle protein synthesis *in vivo* in type 1 diabetic subjects with a biopsy technique suggests that there is no effect of insulin on muscle protein synthesis when hypoaminoacidaemia develops (Pacy *et al*, 1989), and when hypoaminoacidaemia is prevented by continuous infusion of a mixed amino acid solution (Chapter 7). It, therefore, appears possible that insulin resistance in type 1 diabetic patients, demonstrated for glucose metabolism and for whole-body proteolysis, extends to amino acid metabolism, particularly protein synthesis, in skeletal muscle.

These studies were designed to assess by tracer exchange the acute effects of insulin and of amino acids, separately and in combination, on both skeletal muscle and whole-body protein turnover and amino acid metabolism in type 1 diabetic patients. In addition an aim was to compare the anabolic effects of insulin in the presence of amino acid sufficiency in diabetic patients and nondiabetic subjects.

8.3 Methods

8.3.1 Subjects

Eight patients with type 1 diabetes were studied (Table 8.1). Their diabetes was maintained under good control with insulin two to four times daily. Seven had no overt diabetic complications and one patient had laser-treated retinopathy. They had no additional illness, their biochemical tests of hepatic, renal and thyroid function were normal and their only medication was insulin. Six were connecting-peptide negative and two were deficient (0.08 and 0.13 nmol/l during

hyperglycaemia and ketosis). Eight non-diabetic subjects (Table 8.1, Chapter 6), matched for age, sex, body mass index and body fat composition (determined from skinfold thickness at four sites (Durnin and Womersley, 1974)), were also investigated with the same protocol: these results are described in detail in (Chapter 6).

Table 8.1. Characteristics of Type 1 Diabetic Patients and Nondiabetic Subjects

	Diabetic Patients (n=8)	Controls (n=8)
Age (years)	32(22-43)	33(23-42)
Sex (Female:Male)	2:6	2:6
Body mass index (kg/m^2)	26.2±1.2	23.6±1.3
Body fat (%)	23.8±2.6	21.5±3.3
Duration of diabetes (years)	10.7±1.8	
Insulin dose (U/day)	61±4.5	
Glycated haemoglobin (%, normal <8.5)	9.4±0.8	

Data are means (ranges) or means±SEM.

No differences existed between diabetic patients and controls.

8.4.2 Protocol

The diabetic patients were investigated on two occasions a minimum of 21 days apart. They were given only half of their usual dose of short-acting and no intermediate or long-acting insulin at 5 pm on the evening prior to each study. On each occasion, after an overnight fast (15 h), they were studied during 180 min of continued insulin withdrawal followed by 180 min of euglycaemic hyperinsulinaemia (the insulin replacement period) (Protocol d, Section 3.10.4, Figure 3.4). In random order, on one study day hypoaminoacidaemia was allowed to develop during insulin replacement and on the other day this was prevented by infusion of a commercial mixed amino acid solution (Synthamin 14, Section 3.3, Table 3.1) given at an increased rate during insulin infusion. The nondiabetic subjects were studied after an overnight fast (15 h) with an identical protocol to that used during the amino acid infusion studies in the diabetic patients (Chapter 6).

A cannula for blood sampling was placed, in a retrograde direction, in a dorsal hand vein, and a second sampling cannula, in an antegrade direction, in a common femoral vein (Section 3.5). A third cannula was placed in a contralateral forearm vein for tracer, amino acid and glucose infusions.

Priming doses of L-[^{15}N]phenylalanine (-AA 2.71±0.13, +AA 2.58±0.10 μmol/kg), L-[1-^{13}C]leucine (-AA 6.62±0.28, +AA 7.29±0.37 μmol/kg) and $NaH^{13}CO_3$ (-AA 1.81±0.11, +AA 2.79±0.10 μmol/kg) were administered and a continuous infusion of a mixture of L-[^{15}N]phenylalanine (-AA 2.94±0.12, +AA 2.76±0.12 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) and L-[1-^{13}C]leucine (-AA 6.36±0.24, +AA 6.84±0.24 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) was established and continued until the end of the studies. A priming dose (51.6

mg amino acids/kg over 10 min) of a commercial mixed amino acid solution (Synthamin 14, Section 3.3, Table 3.1) was administered at the beginning of the study of amino acid infusion; Synthamin 14 was then infused until the end of the period of insulin withdrawal at a rate of 0.52 $ml \cdot kg^{-1} \cdot h^{-1}$, providing 44.2 mg of mixed amino acids$\cdot kg^{-1} \cdot h^{-1}$, 15.0 µmol phenylalanine$\cdot kg^{-1} \cdot h^{-1}$, 24.6 µmol leucine$\cdot kg^{-1} \cdot h^{-1}$ and 0.17 g nitrogen$\cdot kg^{-1} \cdot day^{-1}$. Then a primed intravenous infusion (Section 3.9.2) of neutral human insulin (Humulin S, Section 3.3) was administered at 0.29 $nmol \cdot m^{-2} \cdot min^{-1}$ (40 $mU \cdot m^{-2} \cdot min^{-1}$) for 180 min (the insulin infusion period). Glucose solution (1 mol/l, Section 3.3) was infused where plasma glucose fell to 5 mmol/l to maintain euglycaemia (5 mmol/l). During the insulin infusion period, the amino acid infusion rate was increased to 1.96 $ml \cdot kg^{-1} \cdot h^{-1}$ providing 167 $mg \cdot kg^{-1} \cdot h^{-1}$ of mixed amino acids, 56.4 µmol phenylalanine$\cdot kg^{-1} \cdot h^{-1}$ and 93 µmol leucine$\cdot kg^{-1} \cdot h^{-1}$. The amino acid infusion rates were chosen following separate set of studies in eight diabetic patients in whom intracellular amino acid concentrations were measured by percutaneous biopsy after 4 h periods of identical amino acid and insulin infusion; in those studies the intramuscular free leucine and total free amino acid concentrations (Table 7.3, Figure 7.1) were lower during insulin infusion than the values during insulin deficiency even though the plasma values (Table 7.2, Figure 7.1) were greater (Chapter 7).

Blood sampling, breath sampling and indirect calorimetry were as described in Chapter 6 (Section 6.3). Amino acid concentrations in sulphosalicylic acid extracts of plasma were determined in duplicate by reverse-phase high performance liquid chromatography (Section 3.13).

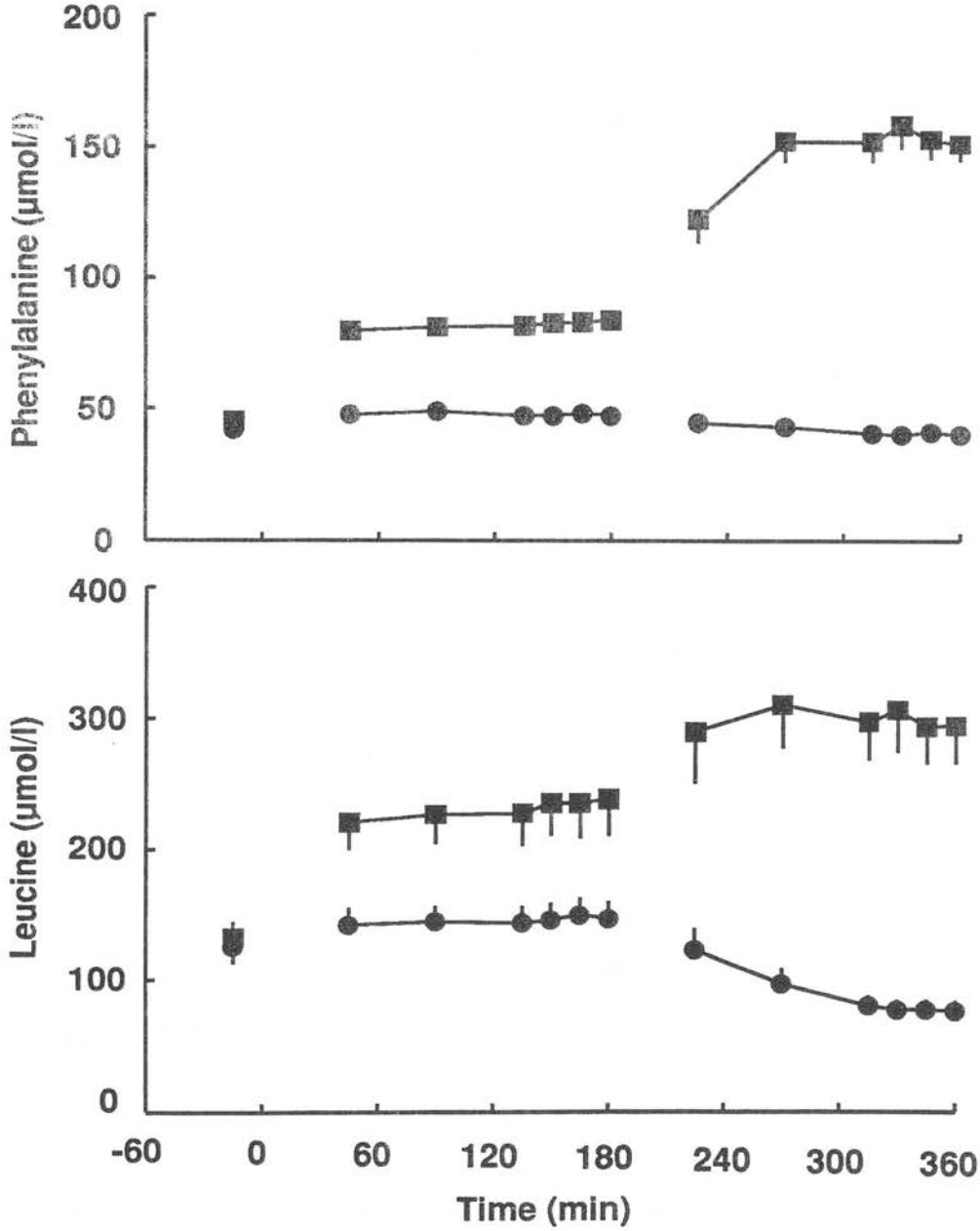

Figure 8.1. Upper panel: Arterial plasma phenylalanine concentrations. Lower panel: Arterial plasma leucine concentrations (circles, without amino acids; squares, with amino acids)

8.4 Results

8.4.1 Gas Exchange and Leg Blood Flow

In the diabetic patients whole-body carbon dioxide production and whole-body oxygen consumption (Table 8.2) were greater during insulin replacement with amino acids than at all other times ($P<0.02$). The respiratory quotient was increased by infusion of insulin and glucose, both during hypoaminoacidaemia ($P<0.02$) and during hyperaminoacidaemia ($P<0.05$). Resting energy expenditure calculated from gas exchange was unchanged by either insulin replacement alone or by amino acid replacement alone but increased ($P<0.02$) during insulin replacement combined with amino acids. In the non-diabetic subjects energy expenditure was not different from the diabetic patients when the results were normalized by expression as $kJ \bullet (kg\ lean\ body\ mass)^{-1} \bullet min^{-1}$ (results not shown). Basal respiratory quotient was not different in the nondiabetic controls but was higher ($P<0.05$) during with insulin plus amino acid infusion. Leg blood flow (Table 8.2) was unaltered by insulin or amino acid infusion: in the nondiabetic subjects blood flow during amino acid infusion alone was not different from that in the diabetic patients but this was faster during insulin plus amino acid infusion (4.74 ± 0.29 *vs* 3.29 ± 0.26 $ml \bullet 100\ g^{-1} \bullet min^{-1}$, $P<0.05$).

8.4.2 Hormones

During insulin withdrawal the plasma free insulin concentration (Table 8.3) was 37 ± 9 pmol/l in the absence of amino acids. Insulin was apparently increased ($P<0.01$) during infusion of amino acids alone despite negative c-peptide assays in

Table 8.2. Indirect Calorimetry and Leg Blood Flow; Effect of Insulin Without (–AA) and With (+AA) Amino Acid Infusion

	Insulin Withdrawn –AA	Insulin Infusion +AA	Insulin Withdrawn –AA	Insulin Infused +AA
VCO_2 (l/min)	0.206±0.008	0.213±0.011	0.214±0.008	0.243±0.011[b]
VO_2 (l/min)	0.284±0.013	0.270±0.017	0.288±0.014	0.312±0.013[b]
Respiratory quotient	0.72±0.01	0.79±0.02[a]	0.75±0.02	0.78±0.02[a]
REE[c] (kJ/min)	5.64±0.25	5.47±0.34	5.74±0.27	6.27±0.26
Leg blood flow ($ml \cdot 100\ g^{-1} \cdot min^{-1}$)	3.26±0.41	3.08±0.43	3.63±0.34	3.29±0.26

Statistical significance: [a]$P<0.05$ effect of insulin replacement, [b]$P<0.02$ different from all other groups. [c]REE, resting energy expenditure.

6 of 8 of the diabetic patients: this anomalous result may possibly have been due to an effect of amino acids on non-specific binding in the insulin assay or may have arisen by chance. During infusion plasma free insulin concentration increased to 529±65 and 561±54 pmol/l in the absence and in the presence of amino acids. Plasma total insulin concentrations were similar in the nondiabetic subjects (Table 6.1). In the diabetic subjects plasma glucagon concentration (Table 8.3) was

decreased 28% ($P<0.01$) by insulin in the absence of amino acid infusion but not in the presence of amino acid infusion. In the nondiabetic subjects plasma glucagon was lower than in the diabetic patients during amino acid infusion alone (34±3 *vs* 50±7 pmol/l, $P<0.05$) but was not different during insulin and amino acid infusion. The concentration of total plasma insulin-like growth factor I was reduced ($P<0.05$) by insulin during amino acid infusion. Similar values were found in the nondiabetic subjects compared to the diabetic patients. Cortisol was unaffected by either insulin or amino acid infusion and was not different in the two groups studied.

8.4.3 Substrates

The arterial plasma glucose concentration in the diabetic patients (Table 8.3) was reduced to a similar extent by insulin in the absence and in the presence of amino acids; during amino acid infusion alone plasma glucose concentration was much lower ($P<0.001$) in the nondiabetic subjects (Table 6.1) than in the diabetic patients but did not differ during the euglycaemic clamp. Whole-body net insulin-stimulated glucose uptake in the diabetic patients was decreased by amino acid infusion (–AA 18.1±4.0 *vs* +AA 14.8±2.9 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, $P<0.05$). In the nondiabetic subjects the rate was higher than in the diabetic patients during amino acid infusion (36.1±4.6 *vs* 14.8±2.9 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, $P<0.01$). Insulin-stimulated leg glucose uptake in the diabetic patients was similar without and with amino acid infusion (–AA 1.46±0.51 *vs* +AA 1.60±0.28 $\mu mol \cdot 100\ g^{-1} \cdot min^{-1}$): in both studies the rate of leg glucose uptake was much higher (–AA $P<0.05$, +AA $P<0.001$) during insulin infusion than was the case during insulin withdrawal when it was not different from zero. In the nondiabetic subjects insulin stimulated leg glucose

Table 8.3. Hormone and Substrate Concentrations; Effects of Insulin Without (–AA) and With (+AA) Amino Acid Infusion

	Insulin Withdrawn –AA	Insulin Infused +AA	Insulin Withdrawn –AA	Insulin Infused +AA
Plasma				
Insulin (pmol/l)	37±9	529±65[d]	61±7[c]	561±54[d]
IGF-I[e] (U/l)	680±60	640±80	680±60	610±70[a]
Glucagon (pmol/l)	48±5	34±5[c]	51±7	50±5
Cortisol (nmol/l)	241±60	276±25	335±72	253±27
Glucose (mmol/l)	10.9±1.5	5.2±0.2[c]	11.8±1.5	6.1±0.7[d]
Blood				
Bicarbonate (mmol/l)	24.4±0.6	26.6±0.3[c]	24.8±0.7	26.4±0.6[d]
Lactate (mmol/l)	0.76±0.05	0.93±0.08[a]	0.63±0.05	0.89±0.06[c]
3-OHBut[f] (mmol/l)	0.68±0.20	0.05±0.02[b]	0.55±0.14	0.10±0.06[c]

Statistical significance: Effect of insulin, [a]$P<0.05$, [b]$P<0.02$, [c]$P<0.01$, [d]$P<0.001$. [e]IGF-I, Insulin-like growth factor I; [f]3-OHBut, D(-)3-hydroxybutyrate.

uptake was faster than in the diabetic patients (3.37±0.52 *vs* 1.60±0.28 μmol•100 g^{-1}•min^{-1}, $P<0.05$).

The arterial blood bicarbonate concentrations in the diabetic patients were only modestly depressed during insulin withdrawal; there was an increase with insulin replacement. The initial arterial blood bicarbonate concentration was higher ($P<0.05$) in the nondiabetic subjects than in the diabetic patients. In the diabetic patients blood lactate concentration (Table 8.3) increased during insulin infusion both in the absence and in the presence of additional amino acids. Net release of lactate by the leg tended to reduce during insulin infusion (–I–AA –0.42±0.16 *vs* +I–AA –0.31±0.10; –I+AA –0.43±0.09 *vs* +I+AA –0.40±0.16 μmol•100 g^{-1}•min^{-1}). Similar concentrations of blood D–(–)–3–hydroxybutyrate were found during insulin withdrawal in the diabetic patients in the absence and presence of amino acid provision; in the nondiabetic subjects (Table 6.1) the concentration was lower ($P<0.05$) during amino acid infusion alone than in the diabetic patients. During insulin infusion D–(–)–3–hydroxybutyrate concentrations in the absence and in the presence of amino acid infusion were similar in the diabetic patients (Table 8.3) and in the nondiabetic subjects.

8.4.4 Amino Acid and Keto Acid Arterial Concentrations

The pre-study arterial plasma concentrations of phenylalanine (–AA 42±3, +AA 45±1 μmol/l) and leucine (–AA 125±12, +AA 132±13 μmol/l) were not different on the two days. At the time of sampling during the initial insulin withdrawal phases arterial phenylalanine, leucine (Figure 8.1) and α–ketoisocaproate (–AA 43.3±4.1 μmol/l) concentrations were stable; these were 73% ($P<0.001$) higher and 60% higher ($P<0.001$) and unchanged (40.1±1.7 μmol/l), respectively, with amino acid infusion during insulin withdrawal. During infusion

of insulin alone phenylalanine concentration decreased ($P<0.001$) by 15% and was stable during the sampling period; leucine concentration decreased 45% ($P<0.001$) and also remained stable during sampling; α–ketoisocaproate concentration decreased 47% ($P<0.001$) to 22.9±1.9 μmol/l. Compared to the insulin withdrawal period, insulin infused with extra amino acids resulted in a 87% increase ($P<0.001$) in arterial phenylalanine concentration, a 27% increase ($P<0.001$) in leucine concentration and a 37% decrease ($P<0.001$) in α–ketoisocaproate concentration; all were stable during final sampling. During amino acid infusion alone the arterial plasma leucine and α–ketoisocaproate concentrations tended to be lower in the nondiabetic controls but during insulin and amino acid infusion there were no differences from the diabetic patients.

8.4.5 Leg Amino Acid and Keto Acid Balance

The leg net fluxes of both phenylalanine and leucine (Table 8.4) were negative during insulin withdrawal. Infusion of insulin alone improved ($P<0.01$) phenylalanine balance and leucine balance followed a similar pattern. During insulin infusion with additional amino acids phenylalanine leg uptake changed ($P<0.05$) and became markedly positive indicating that leg protein synthesis exceeded protein breakdown; leucine balance also became positive but the leg α–ketoisocaproate balance became markedly negative at the same time.

8.4.6 Enrichments of Phenylalanine, Leucine and Leucine Metabolites

Plateaux were attained for the enrichments of plasma phenylalanine and leucine (Figure 8.2) and for plasma α–ketoisocaproate and whole-blood and expired

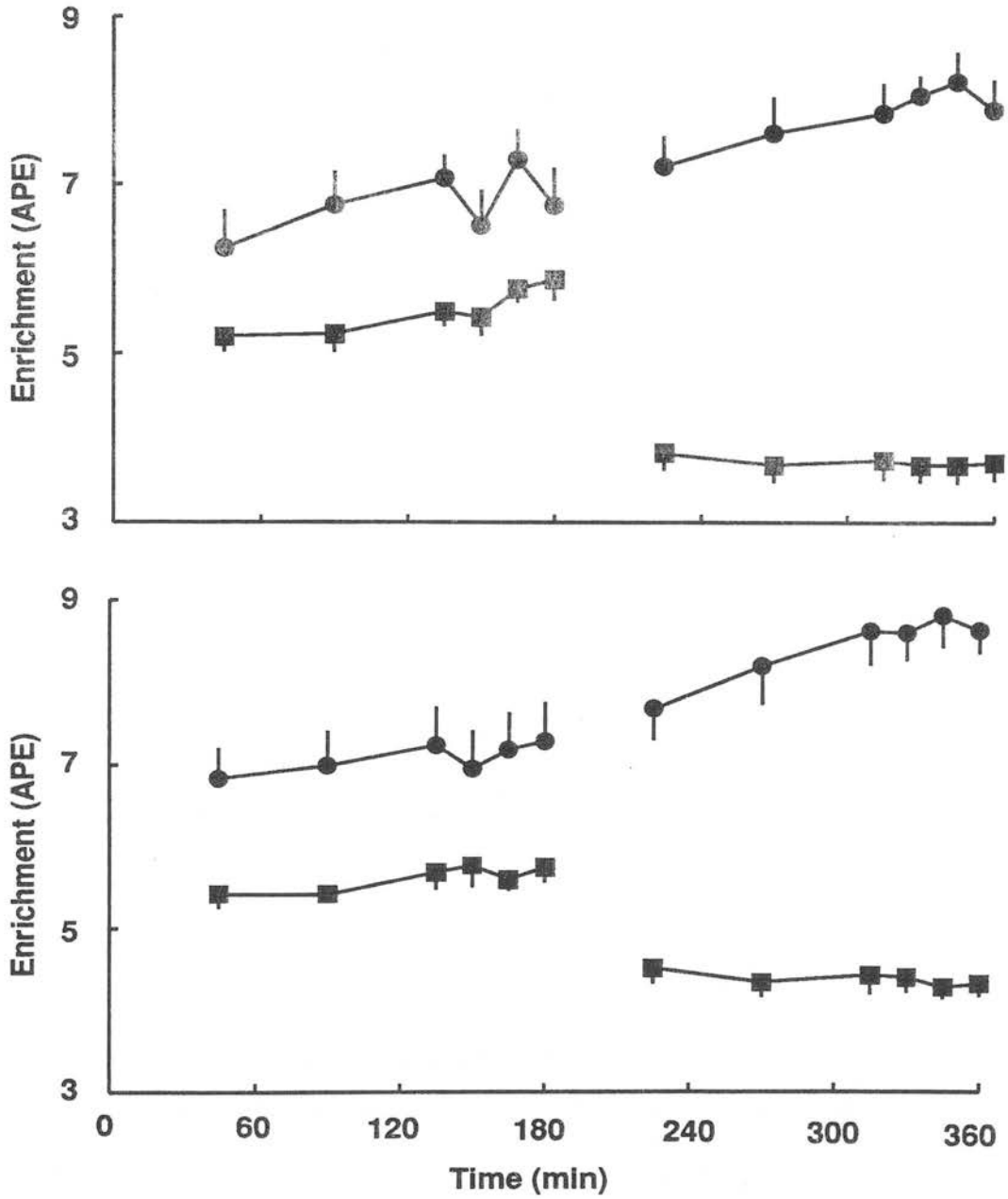

Figure 8.2. Upper panel: Arterial plasma phenylalanine ^{15}N enrichment. Lower panel: Arterial plasma leucine ^{13}C enrichment (circles, without amino acids; squares, with amino acids)

CO_2 (not shown) during the final periods of each phase of study. During insulin withdrawal the ^{13}C enrichment of expired CO_2 was 0.016±0.001 Atoms Percent Excess (APE) both without and with amino acids. During insulin infusion this increased (both $P<0.001$) to 0.022±0.002 and 0.025±0.001 APE, respectively. In three subjects who did not receive tracer (Chapter 6) the ^{13}C enrichment of expired CO_2 was 0.0008±0.0004 APE during insulin, glucose and amino acid infusion suggesting that whole-body leucine oxidation may have been overestimated by about 3% during this period. The leg production rate of $^{13}CO_2$ was not different from zero in the three subjects who received no tracer (Chapter 6) suggesting that a systematic error in the estimates of leucine oxidation, due to changes in substrate metabolism by the leg, did not occurred.

8.4.7 Leg Protein Turnover and Amino Acid Metabolism

Compared with the insulin withdrawal state, insulin infusion alone reduced ($P<0.05$) protein breakdown assessed with [^{15}N]phenylalanine by 42% but assessed with [1-^{13}C]leucine exchange the effect was only a 20% reduction ($P=0.18$, non-significant) in the diabetic patients. There was no effect of insulin alone on protein synthesis (assessed with phenylalanine or leucine) or leucine oxidation by leg tissues.

Compared to insulin withdrawal without amino acids, when amino acids were infused in the diabetic patients protein synthesis assessed with phenylalanine was 66% higher ($P=0.077$); a similar pattern was seen with leucine (+54%, $P<0.05$). No change occurred in the rate of muscle protein breakdown, leucine oxidation or net protein balance as a result of amino acid infusion alone. In the nondiabetic

subjects the rates of leg protein breakdown, synthesis and leucine oxidation during infusion of amino acids alone (Table 6.4) were not different from these in the diabetic patients.

In the diabetic patients infused with amino acids during insulin withdrawal the effect of insulin with additional amino acids was a 41% reduction ($P<0.05$) in muscle protein breakdown assessed with phenylalanine and 32% ($P<0.05$) assessed with leucine. A trend was seen to increased muscle protein synthesis assessed by phenylalanine (+38%, $P=0.07$). No such effect on muscle protein synthesis was observed with the leucine model and the increased leucine balance was largely accounted for by a four fold increase ($P<0.02$) in leucine oxidation by the leg. In the nondiabetic subjects during insulin infusion with additional amino acids muscle protein synthesis assessed by phenylalanine exchange was 60% faster ($P<0.05$) than in the diabetic patients (73.1±7.3 *vs* 45.7±7.5 nmol•100 g^{-1}•min^{-1}). A similar pattern was shown in the leucine exchange based rates which were 54% higher in the nondiabetic subjects (146±21 *vs* 94.8±21.9 nmol•100 g^{-1}•min^{-1}, $P=0.112$). The rates of protein breakdown and leucine oxidation in muscle were not different from the diabetic patients in the nondiabetic control subjects.

8.4.8 Whole-Body Leucine and Phenylalanine Kinetics

In the diabetic patients the effect of insulin alone was to reduce whole-body protein breakdown by 13% ($P<0.001$) and whole-body protein synthesis by 20% ($P<0.001$) assessed with leucine (Table 8.5). Insulin alone induced a 18% increase ($P<0.01$) in whole-body leucine oxidation and a 18% deterioration in the negative

balance between synthesis and breakdown. Whole-body protein breakdown assessed with phenylalanine decreased 19% ($P<0.01$).

Amino acid provision without insulin in the diabetic patients reduced whole-body protein breakdown by 19% assessed with phenylalanine ($P<0.02$) but not with leucine. Increases occurred in whole-body protein synthesis (+11%, $P<0.05$) and in leucine oxidation (+32%, $P<0.01$) and the net balance between protein synthesis and breakdown ($P<0.001$), all assessed with [1-^{13}C]leucine. In the nondiabetic subjects studied during amino acid infusion alone indices of whole-body protein turnover (Table 6.5) were not different from these in the diabetic patients.

In the diabetic patients the effect of insulin with increased amino acid infusion was a 27% reduction ($P<0.01$) in the rate of whole-body protein breakdown, a two fold increase ($P<0.001$) in whole-body leucine oxidation and no change in the rate of whole-body protein synthesis assessed with leucine. The net balance between whole-body protein synthesis and breakdown was improved ($P<0.001$) and became markedly positive during the combined infusion. In the nondiabetic subjects during the combined insulin with amino acid infusion whole-body protein synthesis was 14% faster ($P<0.05$) than in the diabetic patients (126±4 *vs* 111±5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$); there were no differences in the rates of protein breakdown or leucine oxidation.

8.5 Discussion

During the insulin withdrawal periods the degree of insulin deficiency was relatively modest on the basis of the glucagon, bicarbonate and β-hydroxybutyrate concentrations in the type 1 diabetic patients. Under these circumstances infusion

Table 8.4. Leg Phenylalanine and Leucine Metabolism; Effects of Insulin Infusion Without (–AA) and With (+AA) Amino Acid Infusion

	Insulin Withdrawn –AA	Insulin Infused +AA	Insulin Withdrawn –AA	Insulin Infused +AA
Phenylalanine turnover (nmol•100 g^{-1}•min^{-1})				
Breakdown	31.1±5.2	18.0±2.1[a]	43.8±7.1	25.9±3.7[a]
Synthesis	19.9±4.8	18.2±2.6	33.1±8.0	45.7±7.5
Balance (Synthesis-Breakdown)	-11.1±3.6	0.2±2.1[c]	-10.8±4.3	19.8±8.6[a]
Leucine turnover (nmol•100 g^{-1}•min^{-1})				
Leucine net flux	-13.3±10.1	1.6±5.9	6.3±15.4	88.0±17.8[c]
KIC[d] net flux	10.2±4.5	–14.9±7.9	1.3±5.6	–24.6±4.1[b]
Breakdown	72.4±10.1	57.8±8.4	104.1±13.9	70.9±8.4[a]
Synthesis	65.8±14.5	46.6±8.8	101.5±17.1[a]	94.8±21.9
Oxidation	3.5±1.1	8.1±3.2	10.3±2.6	40.8±8.5[b]
Balance (Synthesis-Breakdown)	-6.6±10.7	-11.2±6.5	-2.6±18.1	23.9±16.9

Statistical significance: Effect of insulin, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.

[d]KIC, α-ketoisocaproate.

of insulin alone in insulin-withdrawn has no acute net anabolic effect on either

skeletal muscle protein turnover or on whole-body protein turnover. This result is in keeping with results from previous studies in diabetic patients (Nair *et al*, 1983; Robert *et al*, 1985; Tessari *et al*, 1986a; Umpleby *et al*, 1986; Nair *et al*, 1987a; Pacy *et al*, 1989; Section 1.8.1). In contrast to the effects of insulin, infusion of amino acids in the insulin withdrawn diabetic patients was anabolic by increasing protein synthesis, assessed with leucine, both in muscle and in lean tissues, similar to the effects seen in nondiabetic patients (Chapters 4 and 5). Compared to infusion of amino acids alone the main effects of insulin, with additional amino acids designed to maintain comparable amino acid availability at a tissue level, was to improve skeletal muscle phenylalanine balance due to reduced muscle protein breakdown; there was only a trend to increased muscle protein synthesis with the phenylalanine-based model and no increase was seen with the leucine-based model. In addition, no increase occurred in whole-body protein synthesis during insulin infusion with additional amino acids.

These results in the diabetic patients are in contrast to the results in the nondiabetic subjects in whom infusion of insulin with amino acids *did* exert an anabolic effect by increasing protein synthesis both in muscle and in lean tissues of the whole-body (Chapter 6). As a result of these differing responses to insulin by the diabetic patients and the nondiabetic subjects the rates of both skeletal muscle protein synthesis assessed with phenylalanine and whole-body protein synthesis assessed with leucine were faster in the nondiabetic subjects. These observations suggest that protein synthesis in patients with type 1 diabetes under the study conditions we used is resistant of to acute stimulation by insulin.

Table 8.5. Whole-Body Leucine and Phenylalanine Kinetics; Effects of Insulin Without (–AA) and With (+AA) Amino Acid Infusion

	Insulin Withdrawn –AA	Insulin Infused +AA	Insulin Withdrawn –AA	Insulin Infused +AA
Leucine kinetics ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)				
Flux	119±6	104±5[d]	137±7	174±6[d]
Infusion	--	--	25	93
Breakdown	119±6	104±5[d]	112±7	82±6[c]
Oxidation	22±2	26±2[c]	29±2	64±2[d]
Synthesis	97±4	78±4[d]	108±6	111±5[d]
Balance (Synthesis-Breakdown)	-22±2	-26±2[c]	-4±2	29±2[d]
Phenylalanine kinetics ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)				
Flux	48.5±2.5	39.5±1.7[c]	52.4±3.4	76.6±6.6[c]
Infusion	--	--	15.0	56.4
Breakdown	48.5±2.5	39.5±1.7[c]	37.5±3.3	20.2±1.7[a]

Statistical significance: Effect of insulin, [a]$P<0.01$, [b]$P<0.02$, [c]$P<0.01$, [d]$P<0.001$.

During infusion of insulin alone changes occurred in the concentrations of amino acids and other metabolites and hormones, eg β–hydroxybutyrate, glucose,

lactate, bicarbonate and glucagon; it is most unlikely that insulin directly reduced protein synthesis. Most of these metabolites altered to a similar extent when insulin was infused with amino acids. It is unlikely that these alterations *per se* would have accounted for the changes in protein metabolism: there is evidence that β–hydroxybutyrate stimulates protein synthesis (Nair *et al*, 1988b) and that acid-base alterations also affect protein turnover (Rodriguez *et al*, 1989). Paradoxically plasma insulin-like growth factor I concentrations decreased during insulin infusion as insulin-like growth factor I is said to be depressed in diabetic ketoacidosis (Glaser *et al*, 1987). The probable explanation is that insulin-like growth factor I is almost totally bound to carrier proteins and the small binding protein concentration is rapidly decreased by insulin (Suikkari *et al*, 1989).

In the studies where insulin was infused alone its apparent lack of effect on protein synthesis, both in muscle and in whole-body, may have arisen in part due to differences between the concentrations and enrichments of the tracee in the accessible vascular compartment and at a tissue level. A reduction was observed in the plasma concentrations of both tracers. As transmembrane transport of amino acids by leg tissues is dependent on their concentrations (Lundholm *et al*, 1987), transport of the tracer between plasma and intracellular water, the site of protein synthesis, may have been reduced during insulin infusion alone. If this had occurred to a substantial extent, and there may have been a greater decrement in enrichment within cells during insulin infusion alone than during insulin withdrawal: as a result protein synthesis may have been substantially underestimated. This effect of insulin, to reduce amino acid concentrations with a resultant underestimation of protein synthesis, is directly opposite to the situation

during infusion of amino acids alone. In contrast under these circumstances, both in the present study and in studies described in previous chapters, increased amino acid transport may have resulted in overestimation of the effects of amino acids on both whole-body and muscle protein synthesis (Sections 4.5 and 5.5). It is not possible to ascertain to what extent this may have occurred, and indeed it may have been to a negligible extent.

It is likely that leucine oxidation was overestimated in the present studies as a result of stimulation of leucine oxidation by insulin in adipose tissue (Frick and Goodman, 1980). Adipose tissue is not a site of amino acid storage and leucine undergoing oxidation there would have originated directly from plasma and would have been more highly enriched than leucine entering protein synthesis which would have been diluted from unlabelled amino acids released from muscle protein. This possibility may explain the discordant results for leg protein synthesis during combined insulin and amino acid infusion obtained with phenylalanine and leucine, ie that muscle protein synthesis tended to increase with insulin when assessed with the former but not the latter tracer, analogous to the situation in nondiabetic subjects (Section 6.5). In addition the possibility that the elevated concentrations of the branched-chain amino acids resulted in competitive inhibition of phenylalanine transport by the muscle L-system transporter and also that both *cis*- and *trans*-stimulation occurred which may explain the discordant results (Section 6.5). The greater increases for phenylalanine than for leucine concentration, with the possibility of increased tracer exchange of phenylalanine, may have resulted in a relative overestimation of the effect of insulin plus amino acids on muscle protein synthesis assessed with phenylalanine (Section 6.5).

The phenylalanine-based model for assessing leg protein synthesis is subject to fewer sources of analytical error and to fewer inherent assumptions than the leucine-based model and is, therefore, more robust. This may have been a particularly important factor in the present studies of diabetic patients as a much greater variation was seen in measures of muscle protein synthesis than was the case in the nondiabetic subjects. This may have been due to clinical factors on account of a heterogeneous response by the diabetic patients to insulin withdrawal and also to laboratory factors. The variation may have obscured clinically significant changes in protein turnover. The possibility exists that the effects of insulin with additional amino acid were due simply to the increased arterial concentrations of amino acids which stimulated protein synthesis directly but this is unlikely. Intramuscular amino acid concentrations were lower in a separate group of type 1 diabetic patients studied with a similar protocol (Chapter 7).

The comparisons between the diabetic patients and the nondiabetic subjects is not subject most of the uncertainties noted above and discussed in detail in sections 4.5, 5.5 and 6.5. However, possible reasons for the differences in the results between the groups need to be considered. There was a trend to increased fat mass in the type 1 diabetic patients (Table 8.1). As protein turnover occurs predominantly in lean tissues normalising protein turnover to total body weight may have led to underestimation of protein turnover in the diabetic patients. The small differences in fat mass are not sufficient to explain the differences in protein turnover between the groups. Increases occurred in leg blood flow in the healthy subjects but not in the diabetic patients. Insulin is shown to increase muscle blood flow by adrenergic mechanisms (Laing *et al*, 1982) but it is not known if type 1

diabetic patients have a diminished muscle blood flow response to insulin. The concentrations of glucagon and cortisol tended to be higher in the diabetic patients than in the nondiabetic subjects. There is little evidence that elevated cortisol would have decreased protein synthesis (Wernerman *et al*, 1989) and there is no evidence that glucagon would have had such an effect at the concentrations found in the present study.

The resistance of protein synthesis to insulin is not unexpected as resistance of glucose metabolism to insulin occurred both in the present study and has been well documented by others (DeFronzo *et al*, 1982; Del Prato *et al*, 1983; Pernet *et al*, 1984). The site of insulin resistance of glucose metabolism predominantly appears to be in muscle (Butterfield and Whichelow, 1964; Hother-Nielsen *et al*, 1987; Beck-Nielsen, 1989; Bogardus, 1989) and, in addition, liver where suppression of glucose release by insulin is deficient (DeFronzo *et al*, 1982).

In contrast to the relative insensitivity of protein synthesis to insulin in diabetic patients, the rates of muscle protein synthesis during amino acid infusion were not different in the diabetic patients and in the nondiabetic subjects when insulin was at basal levels, a finding in keeping with the relatively normal glucose-stimulated glucose uptake in type 1 diabetic subjects.

The respiratory gas exchange ratio in the diabetic patients was lower than in the nondiabetic subjects during insulin infusion with amino acids indicating that glucose oxidation was reduced and lipid oxidation was increased. However, plasma β–hydroxybutyrate concentrations were not different in the two groups during insulin infusion, an observation which in contrast does not support the possibility that lipid metabolism was insulin resistant in these diabetic patients.

Although lipolysis is exquisitely sensitive to suppression by insulin there is a relative insensitivity in patients with type 1 diabetes (Jensen *et al*, 1989).

Tessari and colleagues (Tessari *et al*, 1986a; Tessari *et al*, 1988) have shown that whole-body proteolysis in patients with type 1 diabetes is resistant to suppression by insulin. This did not appear to be the case in our study but our study conditions and protein turnover models were different and we infused amino acids with insulin. Therefore, direct comparisons are not possible between these studies.

In summary amino acid stimulation of protein synthesis in muscle and whole-body was normal in patients with type 1 diabetes. In contrast insulin stimulation of protein synthesis, in the presence of a sufficiency of amino acids, was reduced suggesting that insulin resistance extends to amino acid metabolism in addition to glucose and lipid metabolism in subjects with type 1 diabetes. Our finds need to be confirmed with further studies employing a combination of pancreatic and adrenal clamp techniques to enable selective manipulation of elements of the hormone and substrate milieu.

Conclusions

The studies described above are subject to a number of prominent uncertainties and limitations. The most important is that serious errors may have arisen by calculation of indices of protein turnover and amino acid metabolism from the tracee enrichments in the accessible vascular compartment. At the present time the evidence supporting this approach is only circumstantial. As discussed in section 4.5, qualitative mistakes may have arisen in the present studies, particularly that the anabolic effects of amino acids on protein synthesis may have been overestimated.

A second problem is that the coefficients of variation of the measures of muscle protein turnover were large. This was due to errors compounding in the calculations based on small arteriovenous differences and also that the degree of insulin deficiency in the hypoinsulinaemic diabetic patients was variable. It is likely that the forearm would have been a better site to study muscle metabolism; at the antecubital fossa it would have been possible to retrogradely cannulate muscle veins rather than sampling drainage from the complete leg as was the case in our studies. It remains to be tested if this approach would have decreased the variation in the results. The coefficients of variation for muscle protein synthesis calculated from tissue enrichments in samples obtained by biopsy were lower than these determined by the arteriovenous exchange technique. However, it may not be valid to directly compare the results from the two techniques as the arteriovenous exchange technique determines protein turnover at a point in time, whereas the biopsy technique gives an integrated result during a period of time.

Additional elements of the studies to be considered are that the time scale of our protocols may not have been optimal to show insulin and/or amino acid effects, and that the relative content of amino acids in the commercial amino acid solution that we used was not ideal as the plasma phenylalanine concentration

increased during the combined insulin and amino acid infusion and, in contrast, the concentration of tyrosine and other amino acids in plasma and intramuscular water decreased.

Despite the qualifications that must be applied to our results we showed that insulin invariably decreased whole-body protein breakdown. The magnitude of the reduction is consistent with the concept that insulin regulates lysosomal proteolysis, which accounts for about 30% of protein breakdown, and has little effect on other systems for proteolysis. The minimal effect that we found by insulin on muscle protein breakdown in healthy subjects is compatible with the knowledge that myofibrillar protein breakdown is not subject to insulin regulation.

Stimulation of protein synthesis by insulin appeared to be dependent on a rapid amino acid supply. Our results suggest that under such conditions insulin is anabolic by increasing protein synthesis both in muscle and in lean tissues of the body. However, the relatively modest effects of insulin on protein synthesis in the nondiabetic subjects and the lack of such effect in the type 1 diabetic patients is consistent with the hypothesis that factors other than insulin must predominate in the regulation of protein synthesis. We can only speculate on the nature of these factors at the present time but they probably include an interaction between insulin and other hormones, the substrate milieu and neural influences.

Our observation that muscle protein synthesis in type 1 diabetic patients is insensitive to modulation by insulin needs to be verified by additional studies in which hormonal influences are regulated by means of a combination of pancreatic and adrenal clamps. The finding does, however, suggest that insulin effects on protein metabolism in type 1 diabetic patients follows the pattern of resistance documented for glucose and palmitate metabolism.

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Published Papers

1. Bennet WM, Connacher AA, Scrimgeour CM, Smith K, Rennie MJ. Increase in anterior tibialis muscle protein synthesis in healthy man during mixed amino acid infusion: studies of incorporation of [1-^{13}C]leucine. *Clin Sci* 1989; **76**: 447-454.

2. Bennet WM, Connacher AA, Smith K, Jung RT, Rennie MJ. Inability to stimulate skeletal muscle or whole body protein synthesis by insulin and glucose during amino acid infusion: Studies in type 1 diabetic subjects with tracer L-[1-^{13}C]leucine. *Diabetologia* 1990; **33** (1):

3. Bennet WM, Connacher AA, Scrimgeour CM, Rennie MJ. The effect of amino acid infusion on leg protein turnover assessed by L-[^{15}N]phenylalanine and L-[1-^{13}C]leucine exchange. *Eur J Clin Invest* 1990; **20**: 37-46.

Increase in anterior tibialis muscle protein synthesis in healthy man during mixed amino acid infusion: studies of incorporation of [1-^{13}C]leucine

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SUMMARY

1. Anterior tibial muscle protein synthesis in seven healthy postabsorptive men was determined from increases in muscle protein bound leucine enrichment during a primed continuous infusion of L-[1-^{13}C]leucine. Biopsies were taken 30 min after the beginning of leucine infusion (when plasma ^{13}C enrichment was steady), 240 min later during continued fasting and again after 240 min of infusion of a mixed amino acid solution which increased plasma total amino acid concentrations by 37%. The mean enrichment of ^{13}C in plasma α-ketoisocaproate was used as an index of the enrichment of the precursor pool for leucine metabolism.

2. Anterior tibial muscle mixed protein synthetic rate during fasting was 0.055 (SD 0.008) %/h and this increased by an average of 35% during infusion of mixed amino acid to 0.074 (SD 0.021) %/h ($P<0.05$).

3. Whole-body protein breakdown (expressed as the rate of endogenous leucine appearance in plasma) was 121 (SD 8) μmol h^{-1} kg^{-1} during fasting and decreased ($P<0.01$) by an average of 12% during amino acid infusion. Leucine oxidation was 18 (SD 3) μmol h^{-1} kg^{-1} during fasting and increased ($P<0.001$) by 89% during amino acid infusion. Whole-body protein synthesis (non-oxidative leucine disappearance) was 104 (SD 6) μmol h^{-1} kg^{-1} during fasting and rose by 13% ($P<0.001$) during mixed amino acid infusion.

4. ^{13}C enrichment of muscle free leucine was only 61 (SD 19) % of that in plasma α-ketoisocaproate and this increased to 74 (SD 16) % ($P<0.02$) during mixed amino acid infusion.

5. The results suggest that increased availability of amino acids reverses whole-body protein balance from negative to positive and a major component of this is the increase in muscle protein synthesis.

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Key words: amino acids, leucine, muscle protein synthesis, protein turnover.

Abbreviations: APE, atoms per cent excess; KIC, α-keto-isocaproate.

INTRODUCTION

Studies of quadriceps muscle protein synthetic rates in man have shown increases during mixed oral feeding [1, 2], and studies in a variety of disease states [2–4] and during leg immobilization [5] have shown reductions from normal in muscle protein synthesis, but the mechanisms remain obscure.

In model-dependent studies of amino acid exchange across human limbs, an apparent increase in protein synthesis but no change in protein breakdown occurred during mixed oral feeding which should elevate insulin secretion [6, 7], but, paradoxically, insulin infusion in fasting subjects not only did not induce an increase in indirect indices of protein synthesis but reduced protein breakdown [8].

The individual contributions of amino acids, energy (i.e. equivalents of carbohydrate and lipids) and other paracrine and endocrine hormones in the control of skeletal muscle protein synthesis in man are not known. It is known that in man, infusion of substantial amounts of either leucine [9] or mixed amino acids [10, 11] increased whole-body protein synthesis but only a mixed amino acid infusion reduced whole-body protein breakdown, and although the branched-chain amino acids and leucine in particular are said to have anabolic effects on animal muscle no data exist for human muscle. We have begun to tackle these problems by examining the effect of amino acid supply on skeletal muscle protein synthesis.

The aim of the study reported here was to apply stable isotope tracer technology to study the effects on the skeletal muscle protein synthetic rate in postabsorptive

man of an intravenous infusion of mixed amino acids without additional fuel substrates or hormones. A subsidiary aim was to assess the contribution of skeletal muscle to whole-body protein turnover and to examine the changes induced by provision of amino acids.

METHODS

Subjects

Seven healthy men [age 24.5–39.5 years; weight 67.8–77.4 kg (101–114% ideal body weight [12, 13])] were studied at the Department of Medicine, Ninewells Hospital and Medical School, University of Dundee. Each subject gave written consent after a full explanation of the study. The study protocol was approved by the Tayside Health Board Committee on Medical Ethics.

Tracers

L-[1-^{13}C]Leucine and sodium [^{13}C]bicarbonate (both 99%) were obtained from Tracer Technologies Incorporated, Newton, MA, U.S.A. Immediately before administration the tracers were mixed in sterile NaCl solution (150 mmol/l) and were sterilized by passage through 0.22 μm filters (Millipore SA, Molsheim, France).

Experimental design

The subjects were studied after a 15 h overnight fast; they received no food throughout the study, during which they were semi-recumbent in a thermoneutral environment (25–27°C). Arterialized venous blood samples were taken from a 1.7 mm outer diameter cannula (Venflon, Viggo AB, Helsingborg, Sweden), placed retrogradely in a dorsal hand vein and maintained patent by slow infusion of NaCl solution (150 mmol/l); before sampling the hand was warmed by insertion into a thermostatic chamber at 75°C [14]. Priming doses of L-[1-^{13}C]leucine [7.17 (SD 0.51) μmol/kg] and NaH^{13}CO$_3$ [1.86 (SD 0.08) μmol/kg] were given, and a constant infusion of L-[1-^{13}C]leucine [7.39 (SD 0.24) μmol h^{-1} kg^{-1}] was established, via a 1.7 mm outer diameter cannula in a contralateral forearm vein; infusions were delivered and controlled with IMED 928 volumetric infusion pumps (IMED Ltd, Abington, Oxon, U.K.). Thirty minutes later (elapsed time 0 min) anterior tibial muscle was biopsied using 6.5 mm Tilley Henckel ethmoid punches (S. Murray and Co, Sheffield, U.K.) via a 10 mm long skin incision made using 5 ml of 20 g/l lignocaine anaesthesia, subcutaneously infiltrated to the fascia [15]. The biopsies were from the most muscular aspect of the skin at a depth of 10 mm below the fascia. Samples were immediately frozen in liquid nitrogen and stored at −194°C until analysis. A second biopsy was taken 240 min later (elapsed time 240 min), from either the ipsilateral leg (40 mm distant from the initial biopsy site) or the contralateral leg. A third anterior tibial biopsy was taken 240 min after the second biopsy (elasped time 480 min), from the leg contralateral to the first biopsy. After the second biopsy was taken a priming dose of a commercial preparation of mixed amino acids (Synthamin 9, Travenol Laboratories Ltd, Thetford, Norfolk, U.K.) was infused intravenously (83.5 mg of amino acids/kg over 10 min), with a second bolus of NaH^{13}CO$_3$ [0.94 (SD 0.03) μmol/kg]; amino acids were then continuously infused until the end of the study at a rate of 1.52 ml h^{-1} kg^{-1} providing 83.5 mg of amino acids h^{-1} kg^{-1}, 46.5 μmol of leucine h^{-1} kg^{-1} and 0.33 g of nitrogen day^{-1} kg^{-1}. Each litre of Synthamin 9 contained the following L-amino acids: alanine 11.38 g, arginine 6.32 g, glycine 5.66 g, histidine 2.64 g, isoleucine 3.30 g, leucine 4.02 g, lysine 3.19 g, methionine 2.20 g, phenylalanine 3.08 g, proline 3.74 g, serine 2.75 g, threonine 3.21 g, tryptophan 0.99 g, tyrosine 0.22 g, valine 3.19 g, total amino acids 55.0 g.

Arterialized venous samples were taken before, 30 min after the start of the tracer infusion (time 0) and every 60 min thereafter for 480 min. Every 60 min, total CO_2 production, total O_2 consumption and resting energy expenditure were determined over 11 min with a ventilated hood, indirect calorimeter [16]. Every 60 min expired breath was collected into a 2 litre plastic bag and an aliquot was transferred into 20 ml evacuated glass tubes (Vacutainer, Becton Dickenson, Rutherford, NJ, U.S.A.) for subsequent determination of ^{13}C enrichment in CO_2 (see below).

^{13}C analysis

Enrichment with ^{13}C in leucine in plasma and neutralized perchloric acid extracts of freeze-dried, ground muscle were determined after the procedure described below. Urea, which otherwise interfered with gas chromatography–mass spectrometry, was removed by incubation (37°C, 15 min) with jack-bean urease; amino acids were extracted (plasma samples only) with methanolic HCl (4:1, v/v), the solvent removed by evaporation, and then the residue redissolved in pyridine before preparation of t-butyldiemethylsilyl derivatives by reaction with *N*-methyl-*N*-(t-butyldimethylsilyl)trifluoroacetamide (Regis Chemical Company, Morton Grove, IL, U.S.A.) [17]. Gas chromatography–mass spectrometry was carried out on a Finnigan 1020B instrument (Finnigan MAT Ltd, Hemel Hempstead, U.K.), fitted with a 20 m × 0.2 mm inner diameter OV-1 WCOT chemically bonded fused silica column (Pierce UK Ltd, Cambridge, U.K.), with temperature programmed from 110 to 270°C at 15°C/min after a 1 min lag. Decane was used as solvent. The injector temperature was 280°C and injection, in the splitless mode, was complete in 30 s. The carrier gas was helium at 50 kPa. The mass spectrometer was operated in the electron impact mode with an ionization energy of 70 eV. [1-^{13}C]Leucine enrichment was determined by monitoring m/z 302 and 303. The enrichment in α-ketoisocaproate (KIC) was determined by analogous methods using *o*-trimethylsilyl quinoxalinol derivates [18] and monitoring m/z 232 and 233.

Enrichment of ^{13}C in expired CO_2 was measured with an automated Finnigan MAT breath gas analysis system attached to a Finnigan MAT Delta D isotope ratio mass spectrometer [19]. To determine the ^{13}C enrichment in

protein-bound leucine, protein from freeze-dried, ground muscle samples was repeatedly washed with perchloric acid, acid-hydrolysed (6 mol/l HCl) and leucine was separated by preparative gas chromatography [20]. Leucine-CO_2 was liberated by ninhydrin and its ^{13}C enrichment determined using the breath gas system optimized for small quantities of CO_2 [21].

Metabolite and hormone concentration assays

Amino acid concentrations were determined in plasma and muscle sulphosalicylic acid or perchloric acid extracts with an amino acid analyser (LC5000, Biotronik GmbH, Munchen, F.D.R.) using ion-exchange chromatography and fluorimetric detection of *o*-phthaldehyde derivatives with norvaline as internal standard. Intracellular free amino acids were extracted from freeze-dried, ground muscle samples with 0.5 mol/l perchloric acid; calculation of the values for intracellular concentration and enrichment was made on the assumption that 13% of the water content, by weight, of the muscle sample was extracellular [22]. Concentrations of plasma glucose were determined with a Glucose Analyser 2 (Beckman Instruments, Irvine, CA, U.S.A.) and of blood D-(−)-3-hydroxybutyrate by an nicotinamide–adenine dinucleotide-linked enzyme assay [23]. Plasma hormone concentrations were measured at 180, 240, 420 and 480 min by radioimmunoassay, insulin by a double-antibody kit (Ire-Medgenix Sa, Brussels, Belgium), glucagon by a double-antibody kit (Radioassay Systems Laboratories Inc., Carson, CA, U.S.A.) and cortisol by a solid-phase primary antibody kit (Immunodiagnostic Systems Ltd, Washington, U.K.).

Calculations

Muscle protein synthetic rates were calculated from the increase in incorporation of L-[1-^{13}C]leucine into the protein-bound leucine pool, assuming that the mean ^{13}C enrichment in plasma KIC from 0 to 240 min and 300 to 480 min reflected that in the precursor pool, in the absence and in the presence of amino acid infusion, respectively [1]. The components of plasma leucine turnover were calculated from the mean plasma KIC enrichment from 60 to 240 min and 300 to 480 min [1]. Leucine oxidation was calculated from whole-body $^{13}CO_2$ excretion and plasma KIC enrichment [1]; during an additional study without tracer ^{13}C, enrichment in expired CO_2 was only 0.00018 (SD 0.00015) atom per cent excess (APE) during amino acid infusion.

Statistical analysis

Mean values and their SD are presented. Results from the two phases of the study were compared with the Student's *t*-test for paired data.

RESULTS

Indirect calorimetry

Resting energy expenditure was 5.42 (SD 0.28) kJ/min in the absence of the amino acid infusion and increased to 5.87 (SD 0.27) kJ/min ($P<0.01$) during the infusion. No change was found in the respiratory quotient (0.73 overall).

Substrates and hormones (Table 1)

Values of the concentrations of plasma glucose, insulin, glucagon and cortisol and of blood D-(−)-3-hydroxybutyrate were stable within each study period, without and with amino acid infusion. During amino acid infusion insulin was 53% greater ($P<0.05$) and glucagon 41% greater ($P<0.01$) compared with postabsorptive concentrations. Concentrations of glucose, D-(−)-3-hydroxybutyrate and cortisol were not affected by the amino acid infusion.

Amino acid concentrations (Fig. 1 and Table 2)

The mean plasma leucine concentration (Fig. 1) in the absence of amino acid infusion, from 0 to 240 min, was 157 (SD 22) μmol/l; a rise ($P<0.05$) of 16% occurred from 60 to 240 min. During amino acid infusion leucine increased by nearly 80% to a plateau value of 278 (SD 46) μmol/l ($P<0.001$) between 300 and 480 min. During fasting the concentration of free leucine in intramuscular fluid was not different from that in plasma at 167 (SD 22) μmol/l; this increased by 52% to 254 (SD 55) μmol/l ($P<0.02$) during amino acid infusion when there was no statistical difference between plasma and intramuscular free leucine concentrations. Total plasma amino acid concentration was 2890 (SD 260) μmol/l initially and a rise ($P<0.001$) of 37% to 3950 (SD 370) μmol/l occurred during amino acid infusion. During infusion no change

Table 1. Substrate and hormone concentrations in blood and plasma in the absence and in the presence of amino acid infusion

Results are means (SD) over time (see the text). Statistical significance: *$P<0.05$, **$P<0.01$.

	Postabsorptive	Amino acid infusion
Blood D-(−)-3-hydroxybutyrate (mmol/l)	0.14 (0.08)	0.16 (0.04)
Plasma glucose (mmol/l)	4.7 (0.3)	4.9 (0.3)
Plasma insulin (m-units/l)	6.2 (4.0)	9.5 (4.4)*
Plasma glucagon (pmol/l)	35.6 (14.5)	50.2 (16.8)**
Plasma cortisol (nmol/l)	279 (99)	249 (204)

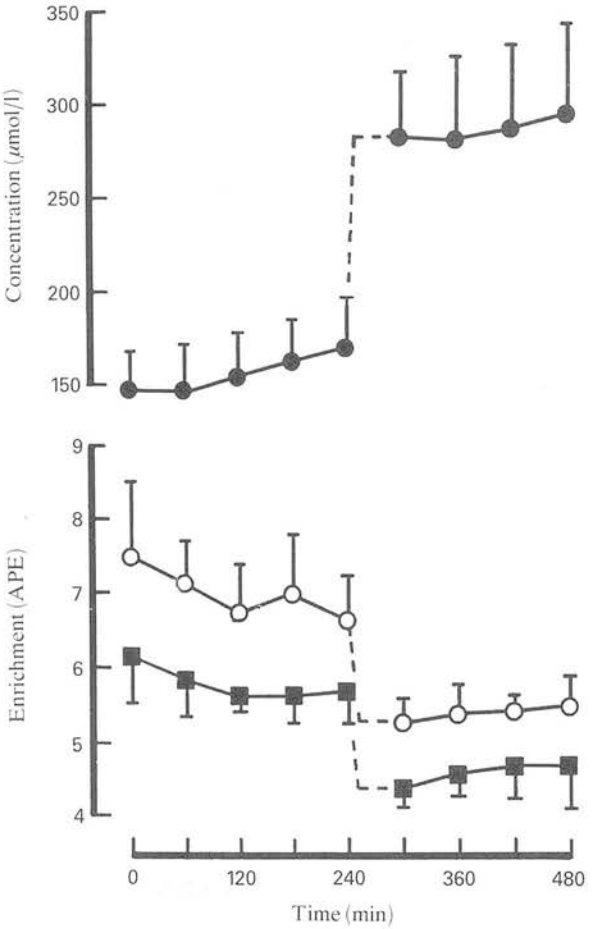

Fig. 1. Plasma concentration of leucine (●) and ^{13}C enrichment of plasma leucine (○) and KIC (■). Results are means with bars indicating SD. L-[1-^{13}C]Leucine was infused in the absence (−30 to 240 min) and in the presence (240 to 480 min) of a mixed amino acid infusion (see the Methods section).

was found in the plasma concentrations of aspartic acid and glutamine; reductions occurred in glutamic acid and taurine, amino acids not present in the mixture infused.

Plasma and muscle free leucine pool enrichments

For both plasma leucine and KIC, plateau enrichment was achieved during the last 180 min of each phase of the study (Fig. 1, Table 3). The ratio of ^{13}C enrichment in plasma KIC relative to that in plasma leucine was 83 (SD 4) % in the absence of exogenous amino acids and this was maintained [85 (SD 7) %] during amino acid infusion. The ^{13}C enrichment in muscle free leucine was 4.26 (SD 1.56) APE 30 min after the [^{13}C]leucine bolus (time 0 min) and was 3.45 (SD 1.10) APE at the end of the first phase of the study before infusion of amino acids. This enrichment was unchanged [3.41 (SD 0.81) APE] during amino acid infusion despite reductions in the enrichment of ^{13}C in both plasma leucine and plasma KIC (Table 3). The ^{13}C enrichment of intramuscular free leucine was 51 (SD 7) % of that of plasma leucine in the absence of exogenous amino acids and rose to 63 (SD 14) % ($P<0.02$) during amino acid infusion. The ^{13}C enrichment of intramuscular free leucine was 61 (SD 19) % of that of KIC in the absence of exogenous amino acids and rose to 74 (SD 16) % ($P<0.02$) during their infusion.

Muscle protein synthesis (Table 4)

The rate of anterior tibial muscle protein synthesis in the absence of amino acid infusion, calculated on the basis that the mean ^{13}C enrichment in plasma KIC (Table 3) should closely represent the precursor synthetic pool and provide a best estimate of protein synthesis, was 0.055 (SD 0.008) %/h; the rate increased during amino acid infusion by 35% to 0.074 (SD 0.021) %/h ($P<0.05$). These values should be considered as the best estimate of protein synthesis. When the synthetic rate was calculated on the basis of plasma leucine enrichment as representative of the precursor pool enrichment, this minimal estimate of muscle protein synthesis was 0.045 (SD 0.008) %/h in the absence of amino acid infusion, 18% slower ($P<0.001$) than the plasma-KIC-based values; a rise of 40% to 0.063 (SD 0.019) %/h ($P<0.05$) occurred during amino acid infusion. These values should be considered as minimal estimates of protein synthesis. When calculated on the basis of the ^{13}C enrichment in intramuscular free leucine taken as representative of the precursor pool enrichment, this maximal or overestimate of muscle protein synthesis was 0.094 (SD 0.044) %/h in the absence of amino acid infusion [71% faster (NS) than the plasma-KIC-based rate]. During amino acid infusion, the rate of muscle protein synthesis calculated in this fashion [0.107 (SD 0.044) %/h] showed no significant difference from that calculated in the absence of exogenous amino acids.

Whole-body leucine kinetics (Table 5)

During the infusion a rise ($P<0.001$) of 26% occurred in the plasma leucine flux, but this increase was less than the exogenous leucine infusion rate because of a reduction ($P<0.01$) of 12% in the rate of appearance of endogenous leucine, a measure of protein breakdown. Whole-body leucine oxidation increased ($P<0.001$), in absolute terms, to almost double the postabsorptive value during amino acid infusion; as a proportion of plasma leucine flux leucine oxidation rose from 15% to 22%. The non-oxidative rate of leucine disappearance, i.e. whole-body protein synthesis, increased ($P<0.001$) by 13% during the amino acid infusion. Net leucine uptake changed ($P<0.001$) from negative to positive, suggesting that net protein balance had become positive.

DISCUSSION

This study shows, for the first time, the effects of parenteral infusion of mixed amino acids on anterior tibial muscle protein synthesis. Information is also provided about the relationships of leucine and KIC in the

Table 2. Amino acid concentrations in plasma and intramuscular water (IMW)

Results are means (SD). Values for plasma and IMW at the same time points are compared, and also values for plasma at different times (increases: $*P<0.05$, $**P<0.01$, $***P<0.001$; reductions: $¶P<0.02$, $¶¶P<0.01$).

	Amino acid concn. (μmol/l)			
	Postabsorptive		Amino acid infusion	
	Plasma (0–240 min)	IMW (240 min)	Plasma (300–480 min)	IMW (480 min)
Alanine	253 (44)	1610 (540)***	467 (66)***	1900 (300)***
Arginine	101 (23)	341 (92)***	211 (46)***	473 (131)***
Aspartic acid	8 (4)	833 (344)***	8 (4)	926 (243)***
Glutamic acid	82 (38)	5210 (520)***	70 (31)¶	5580 (920) ***
Glutamine	673 (44)	11700 (2300)***	658 (57)	12100 (1900)***
Glycine	237 (33)	1450 (480)***	405 (43)***	1600 (290)***
Histidine	92 (11)	369 (54)***	138 (21)***	412 (46)***
Isoleucine	55 (7)	59 (17)	160 (16)***	145 (27)
Leucine	157 (22)	167 (32)	278 (46)***	254 (55)
Lysine	181 (49)	616 (119)***	241 (54)***	679 (166)***
Methionine	19 (2)	—	63 (6)***	—
Phenylalanine	54 (10)	85 (23)**	103 (19)***	132 (20)*
Serine	124 (14)	833 (344)***	186 (14)***	926 (243)***
Taurine	71 (13)	20400 (2900)***	60 (9)¶¶	19800 (3300)***
Threonine	128 (18)	573 (174)***	181 (18)***	641 (101)***
Tryptophan	44 (8)	4870 (2800)***	65 (13)***	7310 (2350)***
Tyrosine	47 (7)	71 (2)*	48 (5)	53 (14)
Valine	254 (48)	511 (142)**	380 (60)***	664 (145)**
Total amino acids	2890 (260)	52400 (7900)***	3950 (370)***	55900 (4300)***

Table 3. ^{13}C enrichment of free leucine and its metabolites in plasma, muscle and breath, and the rate of increase in muscle protein-bound leucine

Results are means (SD). Statistical significance: $*P<0.001$.

	Postabsorptive (0–240 min)	Amino acid infusion (300–480 min)
Plasma leucine (APE)	7.03 (0.56)	5.41 (0.20)*
Plasma KIC (APE)	5.80 (0.33)	4.60 (0.36)*
Muscle free leucine (APE)†	3.45 (1.10)	3.41 (0.81)
Expired CO_2 (APE)	0.0129 (0.0019)	0.0185 (0.0023)*
$10^3 \times$ Protein leucine/time (APE/h)‡	3.20 (0.50)	3.41 (0.93)

†Muscle free leucine results were obtained at either 240 or 480 min of elapsed time.

‡The rate of increase in enrichment of protein-bound leucine was calculated from changes calculated over 0–240 min and 240–480 min.

Table 4. Calculated values of anterior tibial muscle protein synthetic rate according to choice of leucine pool to represent the precursor pool for protein synthesis

Results are means (SD). Statistical significance: $*P<0.05$. Abbreviation: NS, not significant.

Precursor pool assigned	Postabsorptive	Amino acid infusion	*P* value
Plasma KIC (%/h)	0.055 (0.008)	0.074 (0.021)	0.05
Plasma leucine (%/h)	0.046 (0.008)	0.063 (0.019)	0.05
Intramuscular leucine (%/h)	0.094 (0.044)	0.107 (0.044)	NS

plasma pool and in muscle intracellular water of anterior tibialis.

In essence, the results show that increases in amino acid availability in plasma and muscle stimulate muscle protein synthesis, calculated on the basis of the enrichments of plasma KIC or leucine, but not when calculated on the basis of intracellular leucine enrichment. The correct interpretation of these results depends on knowledge of the relative tracer labelling of muscle leucine tRNA, plasma leucine, intramuscular free leucine and the keto-acid of leucine, information not available for man. In previous studies of human muscle protein synthesis [1–5]

Table 5. Whole-body leucine kinetics

Results are means (SD). Statistical significance: $^{*}P<0.01$, $^{**}P<0.001$.

	Rate (μmol h^{-1} kg^{-1})		Change (%)
	Postabsorptive	Amino acid infusion	
Infusion rate	—	46.5	—
Flux	121 (8)	153 (13)**	+26
Protein breakdown	121 (8)	106 (13)*	−12
Oxidation	18 (3)	34 (6)**	+88
Protein synthesis	104 (6)	118 (8)**	+13
Net protein balance	−18 (3)	+12 (6)**	

we used the plasma leucine or KIC enrichments as the basis of our calculations, since six studies [24–29] of precursor pool labelling of leucine in skeletal and cardiac muscle, both *in vitro* and *in vivo*, showed tRNA labelling to be nearer to that of the extracellular, than to that of the intracellular, amino acid pool, but one other study reports the opposite result [30]. The substantial transamination of leucine in human forearm arteriovenous exchange studies [6, 7] provides further evidence of rapid entry of extracellular leucine to intracellular metabolic processes.

The relative increase in the equilibrium between the intramuscular free leucine enrichment and that of plasma leucine during amino acid infusion is partly a function of the reduced arteriovenous decrement in leucine enrichment that occurs when plasma leucine concentration is increased [6, 7, 31] and partly to the increased flux of leucine [6, 7] in the fed state. The initial (time 0 min) higher intracellular leucine enrichment may have been a transient effect due to the bolus of [1-^{13}C]leucine or to an increase in protein breakdown during the initial period of the study. It remains a possibility that the lack of a significant effect of amino acid infusion on muscle protein synthesis, calculated on the basis of the free muscle leucine labelling, was a type 2 error: this may have occurred as a result of taking an insufficiently long period over which to measure the enrichment of muscle protein, or to the effect of the relative methodological precision of measurements made on small samples of free and protein-bound intramuscular leucine with a resulting greater variability of the calculated protein synthetic rates. Until information is available on the leucyl-tRNA charging of human skeletal muscle, rates of muscle protein synthesis derived from plasma KIC enrichment should be viewed as the best available estimates, rates derived from plasma leucine enrichment as minimal estimates and rates derived from intracellular leucine enrichment as overestimates.

Complete mixtures of amino acids are reported to enhance protein synthesis in cell-free systems, suggesting that there is a direct stimulatory effect of amino acids on protein synthesis [32]. There is, however, no known mechanism for this effect and the K_m for the charging of tRNA is likely to be much lower than the intramuscular concentration of amino acids so that tRNA is always fully saturated [32]. Leucine has been reported to stimulate rat muscle protein synthesis *in vitro* [33] and whole-body protein synthesis in man [9], but this may occur by indirect and unknown mechanisms. Branched-chain amino acids have no effect on muscle amino acid balance [34] or ribosome profile in man [35]. However, our results showed that amino acid infusion caused an increase in intramuscular leucine and other branched-chain amino acid concentrations so that if they were anabolic there was scope for the effect to be expressed.

To what extent are the increases in muscle protein synthesis likely to result from the small increases in insulin? Many studies in animals both *in vitro* [29, 36, 37] and *in vivo* [38, 39] provide evidence of a stimulatory effect of insulin on muscle protein synthesis, most sensitive in the range 0–15 m-units/l [40]. Studies in adult man conflict with these animal-based results, suggesting that insulin may not have an anabolic effect [8, 41]. An increase occurred in glucagon but this hormone certainly does not stimulate muscle protein synthesis at the concentrations found and may decrease it at pathophysiological concentrations [42].

It remains possible that growth hormone itself induced the reported changes as this hormone is shown to have an acute, direct insulin-like effect on rat muscle protein synthesis *in vitro* [43]. Insulin-like growth factors, expressed by messenger ribonucleic acids in most human foetal tissues, including muscle [44], may have exerted a paracrine influence to modulate increases in protein synthesis in response to amino acid provision, but in identical studies we were unable to measure any change in plasma insulin-like growth factor 1 concentration in response to an identical infusion of amino acids [basal 790 (SD 240) versus amino acids 790 (SD 220) units/l, $n=5$]. Since the anabolic effect of the exogenous amino acids on muscle cannot be firmly identified with changes in availability of insulin, glucagon (or cortisol, glucose and ketone bodies, none of which changed in concentration), we are therefore forced to the conclusion that either the simple increase in intramuscular concentration of amino acids or an increase in some other unknown anabolic factor caused the observed increase in muscle protein synthesis. The present results strongly suggest that a major component in the twofold increase seen in muscle protein synthesis in going from the postabsorptive to the fed state [1, 2] is simply due to an increased availability of amino acids.

The values in the postabsorptive state for anterior tibialis muscle mixed protein synthesis are somewhat higher than those for quadriceps [0.046 (SD 0.012) %/h]

obtained with identical methods and methods of calculation, by ourselves and other workers [2, 5]. This difference may be due to the greater type 1 fibre content of anterior tibial muscle [73 (SD 10) %] compared with quadriceps [47 (SD 9) %] [45]. At present the relative synthetic rates for human type 1 and type 2 muscle fibres are not known, but it is known that in animals muscles with predominantly type 1 fibre composition turn over faster than those with predominantly type 2 [36, 42, 46].

The absolute values, and the changes that occurred between the postabsorptive and amino acid supplemented states in the plasma fluxes of leucine and KIC, and the rates of oxidation of leucine, are in accord with these expected from previous work [10, 11]. The most important feature of the results for the present discussion is the net anabolic effect of exogenous amino acids due to an increase in non-oxidative leucine disappearance (whole-body protein synthesis) and a reduction in the appearance of leucine from protein breakdown.

Given the fact that skeletal muscle contributes about 50% of lean body mass [47], it is possible to calculate (assuming that muscle contains 180 mg of protein/g, protein contains 8% leucine and all skeletal muscle is similar to anterior tibialis) the contribution of muscle to whole-body protein synthesis (non-oxidized leucine disappearance): it would be 24 μmol h^{-1} kg^{-1} in the postabsorptive state and 33 μmol h^{-1} kg^{-1} during exogenous amino acid infusion. These estimates would be reduced by 16% if skeletal muscle turnover generally reflected that of quadriceps. Such calculations suggest that in the postabsorptive state skeletal muscle may account for between 20 and 24% of whole-body protein synthesis. The relative change, on addition of exogenous amino acids, was twice as great for anterior tibialis than for the whole-body protein mass, suggesting that muscle increased its contribution; indeed calculations show that the proportion of whole-body protein synthesis due to muscle rose to between 23 and 28%. This is less than the contribution previously calculated [1] because of the revised, lower values now routinely obtained for skeletal muscle protein synthesis [2, 5].

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