DIETARY PROTEIN, PHYSIOLOGICAL CONDITION AND METABOLIC AMINO ACID UTILISATION

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DIETARY PROTEIN, PHYSIOLOGICAL CONDITION AND METABOLIC AMINO ACID UTILISATION

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
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STELLINGEN

1. De metabole benutting van aminozuren na een maaltijd wordt vooral bepaald door het eiwitgehalte van de maaltijd en in veel mindere mate door de fysiologische toestand van het dier.
   Dit proefschrift.

2. Gezien de grote variatie in de oxidatie van aminozuren over de dag, kan een korte (30 min) oxidatie meting bij verschillende opname niveaus niet gebruikt worden voor het schatten van de behoefte aan aminozuren.
   Dit proefschrift.
   Young, V.R., Bier, D.M. & Pellett, P.L. (1989) A theoretical basis for increasing current estimates of the amino acid requirements in adult man, with experimental support. *American Journal of Clinical Nutrition* **50**, 80-92.

3. Het geringe verschil in de metabole benutting van leucine na een maaltijd tussen groeiende en volwassen dieren ondersteunt de hypothese van Millward dat in beide situaties het voedingseiwit zoveel mogelijk moet worden vastgelegd.
   Dit proefschrift.
   Millward, D.J. & Rivers, J.P.W. (1988) The nutritional role of indispensable amino acids and the metabolic basis for their requirements. *European Journal of Clinical Nutrition* **42**, 367-393.

4. Het niet-lineaire verband tussen de eiwitopname en de stikstofbalans bij volwassen dieren kan worden verklaard door een hogere oxidatie van aminozuren na een maaltijd bij een toenemende eiwitopname.
   Dit proefschrift.
   Munro, H.N. (1985). Historical perspective on protein requirements: objectives for the future. In: *Nutritional Adaptation in Man*, pp 155-168 [K. Blaxter and J.C. Waterlow, editors]. London: John Libbey Publishers.

5. Het optimaliseren van de metabole benutting van aminozuren na een maaltijd zal leiden tot een verbetering van de voedingstoestand van de volwassen mens, een verhoging van de efficiëntie in de dierlijke produktie en een vermindering van de stikstofbelasting van het milieu.
   Dit proefschrift.
   Nationale Raad voor Landbouwkundig Onderzoek (1990) *Meerjarenvisie Landbouwkundig Onderzoek 1991-1994.* Den Haag: NRLO.

6. Omdat de voordrachten van het wetenschappelijk personeel (WP) steeds vaker worden verzorgd door de AIO's, is het niet altijd duidelijk wie het goede voorbeeld geeft.

7. "Bij ieder wetenschappelijk voortbrengsel is de omgeving, waarin men werkt, van groot belang en een deel der verkregen uitkomsten is te danken aan die invloed"
   Curie, E. (1955) *Madame Curie.* 25ste druk. Den Haag: H.P. Leopolds Uitgeversmij.

8. Nationalisten die tegen 'Europa' zijn, kijken liever niet te ver terug in hun eigen geschiedenis.

Aanbevolen literatuur, uit de serie *The Peoples of Europe*: James, E. (1991) *The Franks*. Oxford: Blackwell.

9. "Als vrouwen net zo worden als mannen is dat slecht voor het milieu".

Weeda, I. (januari 1993) *Milieudefensie*.

10. De computer is een uitdaging voor de mens.

11. Uitgaande van de kosten, speelt het feest een belangrijke rol bij een promotie.

Peter J.M. Weijs
Dietary protein, physiological condition and metabolic amino acid utilisation
Wageningen, 16 februari 1993

Vakgroep Fysiologie van mens en dier
Landbouwuniversiteit Wageningen

UITNODIGING
SPECIAAL COLLOQUIUM

Protein turnover in health and disease

Peter J. Garlick
Rowett Research Institute
Aberdeen, Scotland

16 februari 1993 (12.30 - 13.30 uur)

Collegezaal Vakgroep Fysiologie van mens en dier
Haarweg 10, 6709 PJ Wageningen

Informatie: Dr. V.V.A.M. Schreurs (83024/84136)

Ter gelegenheid van de promotie van Peter Wolfe op het proefschrift *Dietary protein, physiological condition and metabolic amino acid utilization* om 16.00 uur in de aula van de Landbouwuniversiteit Wageningen, Generaal Foulkesweg 1a, Wageningen.

"All our knowledge has its origins in our perceptions."

"Knowledge, the natural desire of all good men."

Leonardo da Vinci.

Aan mijn ouders

Aan Saskia

ABSTRACT

Weijs, P.J.M. (1993) Dietary Protein, Physiological Condition and Metabolic Amino Acid Utilisation. PhD Thesis, Department of Human and Animal Physiology, Wageningen Agricultural University, Wageningen, The Netherlands.

This thesis describes the investigated effects of the level of dietary protein intake and the physiological condition of the animal on the percental oxidation of leucine. This measure reflects which part of the free leucine pool was used for protein and energy metabolism. The employed technique consisted of a constant infusion of ^{14}C-leucine and simultaneous collection of expired $^{14}CO_2$. The aim of the study was to identify the causes and objectives of amino acid losses and therefore the metabolic basis for their requirements. The percental oxidation of leucine was decreased in the postabsorptive state by chronic protein restriction, growth and pregnancy, and was increased by a period of fasting, meal frequency and exercise. The transition from the postabsorptive to the postprandial state caused an increase in expired $^{14}CO_2$. The percental oxidation in the postprandial state increased with the protein content of the meal. During different phases after the meal the percental oxidation was decreased by growth and pregnancy (2-4 h), chronic protein restriction (4-6 h), and training (6 h) and increased by the period of fasting (6 h). Several other aspects, such as the level of leucine intake, the use of tracer isotopomers, duration of tracer infusion, and the route of tracer administration were investigated. From these studies it was concluded that there is a large diurnal variation in amino acid losses, which have to be accounted for in the estimation of nutritional requirements.

Amino acid metabolism: Dietary protein: Physiological condition: Leucine: Rat

CONTENTS

"The value of a theory is not measured by its length of life, but rather by the work it stimulates" Carl Voit (Beach, 1948)

GENERAL INTRODUCTION

PROTEIN AND AMINO ACID METABOLISM IN RELATION TO REQUIREMENTS

From nutritional studies with dogs Magendie (1783-1855) was the first to conclude that nitrogen containing foodstuffs are an essential component of the diet. He was also the first to recognise that body constituents are not constant but are continuously renewed (Munro, 1964). Later theories on protein metabolism of von Liebig (1803-1873), Voit (1831-1908) and Folin (1905) however did not use his ideas (**Figure 1**). Liebig was convinced that all dietary protein became first part of structural body protein and could then be converted to nitrogen. Thus, the excreted nitrogen directly reflected body protein breakdown and therefore protein requirements. Voit thought that there was a plasma or labile protein that was converted either to nitrogen or to structural proteins. This hypothesis of two protein pools culminated with Folin who thought that the excretory products indicated which protein, the plasma or the structural protein, was catabolised. He concluded that maintenance protein requirements were very low because only a small part of the excreted nitrogen had been derived from structural protein. These authors could only study body protein as a 'black box', with nitrogen supply with the diet and nitrogen leaving the body with waste products. The nitrogen-balance technique, originally used by Boussingault (1802-1887) and later perfected by Voit, was a fine tool to study the black box (Beach, 1948; Munro, 1964). Much later Schoenheimer *et al.* (1939) investigated "*whether in nitrogen equilibrium the nitrogen in the urine is derived from the food proteins directly or whether dietary nitrogen is deposited, with liberation of an equivalent amount of tissue nitrogen for excretion*". They kept a mature rat (15 months, 350 g) for 10 days on a 15% casein diet which was supplemented with ^{15}N-tyrosine, and showed that only 50-60% of ^{15}N was excreted with the urine, and the remainder was incorporated in tissue protein. For the first time it had been demonstrated that the body could be in nitrogen

equilibrium and at the same time dietary nitrogen could be converted to body protein and the remaining part excreted in the urine. Recognition of the existence and importance of protein turnover was a milestone in the study of protein metabolism (Waterlow *et al.* 1978). A simple two-pool model, that recognised both the free amino acid pool and body protein pool, was developed.

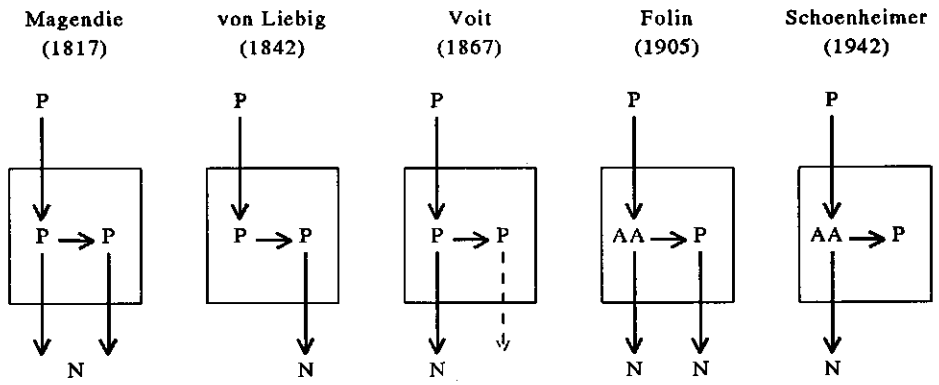

Figure 1. Different views of nitrogen (N), protein (P) and amino acid (AA) metabolism. See text for details.

Since the recognition of the essential nature of nitrogen as dietary component, many views have been discussed but it still remains a problem to quantify protein requirements (Munro, 1985). Information about protein requirements has mainly been based upon the nitrogen-balance technique. Although this technique has provided very important information, it also has several disadvantages (Hegsted, 1976; Munro, 1985; Young, 1986). Since nitrogen equilibrium can be achieved at different levels of protein intake, it is probably not a good criterion of requirement. Unfortunately a good functional indicator of requirement is not known. Therefore current estimates of protein and amino acid requirements are still based on nitrogen balance studies (FAO/WHO/UNU, 1985). At present it seems doubtful that any single value can account for daily protein and amino acid requirement.

Since Schoenheimer *et al.* (1939), protein turnover studies have primarily benefited from the early development of the nitrogen and later the amino acid flux method (Sprinson & Rittenberg, 1949; San Pietro & Rittenberg, 1953; Waterlow, 1967; Waterlow & Stephen, 1967; Waterlow, 1967; Picou & Taylor-Roberts, 1969). Although many investigators have perfected the flux method in recent years (e.g. Matthews *et*

al. 1980; Thompson *et al.* 1989), it remains essentially the same method. For protein synthesis another useful method, the 'flooding dose', has been developed for the rat (Garlick *et al.* 1980) and recently for men (Garlick *et al.* 1989b; Essen, 1991). Protein breakdown is still more difficult to measure. But at least it is now possible to gather information about both protein synthesis and protein breakdown. But since the optimum level of protein turnover is not known, it cannot be used as a criterion for protein requirements.

The optimum level of protein turnover is likely to depend on the desired "end-product". For the adult human the highest possible level of protein turnover might be desirable for long term health, while for (those concerned with) the short living production animal protein turnover may only increase the (amino acid) costs of the end-product and excretion of waste products (environmental N-pollution).

Originally the ^{15}N-labelled amino acids were used in order to measure nitrogen flux, but these studies lasted too long (60 h) to study short term effects. Later the ^{14}C-labelled amino acids and more recently the ^{13}C-labelled essential amino acids were used, and nitrogen flux studies replaced by studies of amino acid flux.

The amino acid flux primarily provides information about the investigated amino acid, and has been used to reestablish the indispensable amino acid requirements. Young and coworkers found current requirements too low (Meguid *et al.* 1986a, 1986b; Meredith *et al.* 1986; Zhao *et al.* 1986; Young, 1987; Young & Bier, 1987; Young *et al.* 1989; Young & Marchini, 1990; Young, 1991). Their work has consequences for protein quality evaluation (FAO/WHO, 1990; Young & Pellett, 1991). But the discussion about the interpretation of these measurements continues (Millward, 1990a; Young *et al.* 1990).

Young and coworkers measured the plateau enrichment of plasma leucine for a period of 30 min in the fed state (Meguid *et al.* 1986). Although they correct their data for the fasted state, no allowance is made for diurnal variation in amino acid oxidation, e.g. after a large meal or exercise. Physiological and/or nutritional conditions can be expected to affect amino acid utilisation and can thus interfere with requirements, but this aspect has hitherto been neglected.

The amino acid flux method has also been used to investigate the effect of feeding and fasting on protein turnover (Garlick *et al.* 1980). The changes in protein synthesis and protein breakdown consequent upon feeding are essential for regulation of dietary amino acid deposition. However it is still controversial whether protein synthesis is increased and/or protein breakdown is decreased (Garlick *et al.* 1990;

Millward *et al.* 1991a). Although the required (possible) increase in protein synthesis and decrease in protein breakdown is not known, both processes contribute to protein accretion after feeding.

Millward & Rivers (1988) have provided a useful model for protein metabolism, in which diurnal protein cycling is of prime importance. Diurnal protein cycling is the result of postprandial protein deposition and postabsorptive amino acid oxidation. Recently Millward *et al.* (1991b) have stressed the importance of measuring the protein deposition efficiency: "*The measurement of the protein deposition efficiency provides a new and important tool for the investigation of the physiological capacity for protein deposition of individual patient groups and for the ability of any particular diet to provide for such deposition. Clearly, since the protein deposition efficiency has not previously been measured in adults on intakes above those required for maintenance, the treatment and dietary regimes which will influence it have yet to be established. It will be particularly important to establish the extent of any defect in those conditions in which impaired maintenance of lean tissue is suspected, during infection, acute or chronic inflammation, in patients on anti-inflammatory steroids, with hormonal abnormalities such as hyperthyroidism, growth hormone deficiency or diabetes, as well as in the elderly.*" Protein deposition efficiency might not only be important in relation to protein loss, but also with protein gain in e.g. meat production animals.

When protein is supplied in an amount equal to the amount of nitrogen excretion on a protein free diet, this will not result in a nitrogen equilibrium. This was described as the non-linear response of the nitrogen balance to protein intake (Munro, 1985). This is not simply an inefficient use of dietary amino acids, but also an inefficient use of endogenous amino acids as a result of dietary supply. Knowledge about the physiological and nutritional factors that influence this utilisation efficiency may result in a more optimal protein and amino acid supply in human, clinical and animal nutrition.

METABOLIC LEUCINE UTILISATION AS A RELATIVE MEASURE

Labelled leucine entering the free leucine pool can either be used for protein metabolism (protein synthesis) or energy metabolism (leucine oxidation), and will be described as metabolic leucine utilisation.
In order to measure absolute values of metabolic leucine utilisation the specific

activity (or enrichment) of the precursor pool has to be measured. It is well recognised that this is a major problem to isotopic studies, in particular in protein metabolism (Waterlow *et al.* 1978).

Since metabolic leucine utilisation is an intracellular event, the intracellular specific activity of leucine would actually have to be measured. Several studies have indicated that plasma specific activity of α-ketoisocaproate (KIC), the keto acid of leucine, can be used as an estimate of intracellular leucine specific activity (Matthews *et al.* 1982; Wolfe *et al.*, 1982; Schwenk *et al.* 1985; Vazquez *et al.* 1986; Layman & Wolfe, 1987; Thompson *et al.* 1988).

However, several organs and tissues are involved in both leucine degradation and incorporation into protein (Abumrad *et al.* 1989; McNurlan & Garlick, 1989), and therefore several specific activities and the extent of their contribution to whole-body leucine utilisation should be investigated, since one tissue may benefit at the expence of another tissue.

Also intracellularly there is compartmentation. Mitochondrial KIC is the immediate precursor for leucine decarboxylation and cytosolic leucyl-tRNA is the precursor for leucine incorporation into protein (Bender, 1985; Stryer, 1988). In vitro studies with cultured skeletal muscle cells have shown that extracellular leucine is preferentially used for leucine decarboxylation (70%), and leucine released from protein breakdown is preferentially used for leucine incorporation into protein (60%) (Sneible *et al.* 1981).

Also intracellular distribution of branched chain amino acid transferase (BCAAT) between cytosol and mitochondria, and the activity state of branched chain keto acid dehydrogenase (BCKA-DH) are tissue dependent (Hutson, 1989).

Therefore the validity of the plasma specific activity is at least arbitrary. At present however the plasma KIC specific activity has been adopted as the most accurate (or the least inaccurate) measure, that can help to estimate rates of leucine flux. The measurement of plasma specific activity, however, requires an isotopic steady state. Therefore measurement of any specific activity in the non-steady state has limited value.

When the prime interest is to study (acute) changes in leucine utilisation as a result of changing nutritional and/or physiological conditions, the non-steady state is essential to the measurements. Since metabolic leucine utilisation can either be leucine decarboxylation and leucine incorporation (not transamination), the main question is whether one is favoured at the expense of the other. This can be described with 'utilisation efficiency', which is expessed as the percentage of leucine utilisation that is

not oxidised.

The efficiency of leucine utilisation does not reflect changes in absolute rates of leucine degradation or incorporation, but preferential use of leucine for one pathway relative to the other. Leucine oxidation studies in the rat have primarily yielded a percental measure of utilisation (**Table 1**). Data from studies in humans however indicate that the relative measure of leucine oxidation (as percentage of leucine flux) is highly correlated (r=0.92) with the absolute measure of leucine oxidation (**Figure 2a**). There is an almost linear relationship between the relative and absolute measure of leucine oxidation between 5% and 25%. The best fit for these data is a log curve, and after log transformation of the absolute values this relationship between the relative and absolute measure is linear (**Figure 2b**).

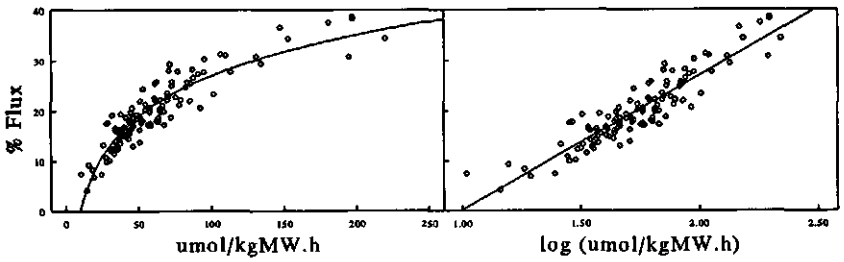

Figure 2. Relationship between the absolute measure (μmol.kgMW^{-1}.h^{-1}; x) and relative measure (% flux; y) of leucine decarboxylation (y = 27 log(x) + 27). Data (n 122) were obtained from 31 studies in mature humans (Bennet *et al.* 1989, 1990, 1991; Clugston & Garlick, 1982; Devlin *et al.* 1990; Garlick *et al.* 1980; Griggs *et al.* 1990; Fukagawa *et al.* 1989; Hoffer *et al.* 1985; Jahoor *et al.* 1989; Matthews *et al.* 1982; Melville *et al.* 1989; Motil *et al.* 1981a, 1981b; Nair *et al.* 1983, 1987a, 1987b, 1987c, 1987d, 1988; Pacy *et al.* 1988a, 1988b, 1989, 1990, 1991; Pelletier *et al.* 1991; Rennie *et al.* 1982; Shangraw *et al.* 1988; Thompson *et al.* 1989; Wolfe *et al.* 1982; Young *et al.* 1987). Left, data plot; Right, linear regression after log transformation of x data.

The effects of different nutritional and/or physiological conditions on metabolic leucine utilisation are probably important on the short term (hours or even less). On the short term it is unlikely that absolute amounts are considered by metabolism, but rather concentrations of leucine. These concentrations are highly relevant to enzyme activity, with the degrading enzymes (BCAAT and BCKA-DH) having a high Km (mM range) and leucyl-tRNA synthetase a low Km (μM range; Harper, 1985). When

leucine concentrations increase, the chance of leucine oxidation increases and the utilisation efficiency decreases.

When daily leucine intake in the mature animal is considered, the absolute amount of daily leucine oxidation is known beforehand (since daily efficiency is zero). Leucine oxidation however is not decided upon on a day to day basis, but on a minute to minute basis. The utilisation efficiency seems an appropriate measure to investigate these short term decisions.

The maximal daily efficiency of dietary leucine depends on the utilisation efficiency after a meal (in the absorptive state). Although the utilisation efficiency will increase thereafter (in the postabsorptive state), the daily or dietary efficiency can only decrease.

On the other hand it is quite impossible to increase the utilisation efficiency upto 100% in the postabsorptive state. Amino acids are precursors for several biosynthetic and oxidative pathways. These pathways continue or become primarily important in the postabsorptive state, e.g. glutamine for the intestine and the immune system and alanine for gluconeogenesis (Cahill, 1970; Snell, 1980; Newsholme, 1988). Leucine can be an important donor of nitrogen for both alanine and glutamine (Snell, 1980; Newsholme, 1988). In the postabsorptive state leucine is available from protein breakdown, but leucine oxidation in the postabsorptive state reduces the (re)utilisation efficiency.

Thus loss of leucine remains apparent in the postabsorptive state, and therefore some dietary leucine has to be stored temporarily (after a meal) in order to account for postabsorptive losses. In fact this holds for most amino acids, since protein deposition is increased after a meal. Postprandial protein deposition and postabsorptive removal is called diurnal protein cycling (Millward & Rivers, 1988).

AIM OF THE STUDY

The aim of the study is to identify causes and objectives of amino acid losses and therefore the physiological or metabolic basis for their requirements.

In this thesis the effect of different nutritional and/or physiological conditions, like acute and chronic protein supply, fasting and feeding, growing and mature status, pregnant and non-pregnant status, meal frequency, training and exercise, on the percental leucine oxidation have been investigated and provide information about major and minor influences on leucine metabolism.

Table I.
Chronic protein restriction and leucine oxidation studies in the rat with different tracers and different routes of administration.

Reference	Animals	Condition	Method	Time h[1]	Diet wks	Protein level	1-^{14}C	U-^{14}C
			Intragastric administration					
Sketcher *et al.* (1974)	growing 5 wks	5 h fast	injection	3	2	10% 3.5%	15.9 4	
Sketcher *et al.* (1974)	growing 5 wks	24 h fast	injection	3	2	10% 3.5%	29 10.9	
Neale & Waterlow (1974)	growing 6 wks	overnight fast	infusion	4	3	20.5% 0%		14.3 15.7
Neale & Waterlow (1977)	200-300 g	no fast	intubation	2		5%	2.96	2.66
Birt & Clark (1976)	220-250 g	6 h fast	oral	4.5	2	6.0% 4.8% 3.6%		4.55 3.71 2.73
			Intraperitoneal administration					
McFarlane & von Holt (1969)[2]	mature 16 wks	14 h fast	injection	3	8	27% 2%	40 10	
Schreurs *et al.* (1991)	mature	no fast	injection	4	3	21% 7.5% 0%	21.0 10.4 6.5	14.4 7.9 6.5
			Intravenous administration					
Neale & Waterlow (1974)	growing 6 wks	overnight fast	infusion	4	3	20.5% 0%		11.9 19.2
Neale (1971)		no fast	injection	1	2	well-fed 1%		9.61 16.9
Neale (1971)		16 h fast	injection	1	2	well-fed 1%		25.5 22.8
Waterlow *et al.* (1978)			infusion	2		25% 5%	42.7 20.4	22.2 12.6

[1] Duration of $^{14}CO_2$ collection. [2] DL-[2-^{14}C]-leucine was used in this study.

Chapter 1

EFFECTS OF ACUTE AND CHRONIC PROTEIN SUPPLY ON METABOLIC LEUCINE UTILISATION IN GROWING AND MATURE RATS

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British Journal of Nutrition (in press)

Effects of acute (meal) and chronic (diet) level of protein supply on metabolic leucine utilisation were investigated in growing (10 wks) and mature (> 1 y) rats. Rats were conditioned on a high protein (HP) diet (210 g casein/kg feed) or a low protein (LP) diet (75 g casein/kg feed) from 7 weeks of age. Overnight fasted rats were offered a HP or LP meal during an 8 h $^{14}CO_2$ breath test with a constant infusion of either L-[1-^{14}C]-leucine (CL) or L-[U-^{14}C]-leucine (UL). Before the meal $^{14}CO_2$ output was lower for overnight fasted rats on a LP diet v. HP diet ($P<0.001$), and also lower for growing v. mature rats ($P<0.001$). Meal ingestion resulted in a fast increase in $^{14}CO_2$ output. From 2 h after start of the meal the effect of acute protein supply on $^{14}CO_2$ output was significant ($P<0.001$), while the effect of chronic protein supply disappeared for CL. After the meal $^{14}CO_2$ output was transiently lower for growing v. mature rats ($P<0.05$), especially after the LP meal. The difference in $^{14}CO_2$ output between CL and UL increased transiently after the meal, indicating an increase in decarboxylation relative to total oxidation of leucine. In conclusion, 1. metabolic leucine utilisation after overnight fasting depends on the level of chronic protein supply and stage of development of the animal, 2. metabolic leucine utilisation after feeding depends primarily on the level of acute protein supply, 3. the transient increase in non-protein label retention suggests a temporal oversupply of leucine relative to the actual metabolic state.

Amino acid metabolism: Leucine: Dietary protein: Growth: Rat

INTRODUCTION

Current estimates of protein and indispensable amino acid (IAA) requirement of adult humans are based on the N-balance technique (FAO/WHO/UNU, 1985). Several authors have stressed the limitations of this technique (Hegsted, 1976; Munro, 1985; Young, 1986). From recent IAA oxidation studies in the fed state at different levels of IAA intake in adult humans, it was concluded that current estimates of IAA requirements were too low (Young *et al.* 1989). There is no consensus however about the metabolic basis for protein and IAA requirements (Millward & Rivers, 1988; Millward *et al.* 1991; Young *et al.* 1991).

Several studies have investigated the effect of feeding and fasting (Garlick *et al.* 1980; Motil *et al.* 1981; Rennie *et al.* 1982; Clugston & Garlick, 1982; Hoffer *et al.* 1985; Melville *et al.* 1989) and the level of chronic protein supply (Millward *et al.* 1991) on metabolic leucine utilisation. These studies however involved steady state periods with or without repeated small meals. The effect of normal intermittent feeding on metabolic leucine utilisation has not been considered.

In the present study the effects of acute and chronic level of protein supply on metabolic leucine utilisation were investigated in growing and mature rats, which have different protein and/or IAA requirements. By means of a $^{14}CO_2$ breath test the meal-induced changes in $^{14}CO_2$ output were measured during infusion of 1-^{14}C- and U-^{14}C-leucine. The difference in $^{14}CO_2$ output between infusion of 1-^{14}C- and U-^{14}C-leucine has been suggested to be an indication of amino acid oversupply relative to metabolic state of the animal (Schreurs *et al.* 1992).

MATERIALS AND METHODS

Animals and diets

Growing (9-10 wks) and mature (> 1 y) male Wistar-WU rats (Centre for Small Laboratory Animals, Wageningen Agricultural University, Wageningen, The Netherlands) were housed in individual cages with bedding material (Woody-Clean type 8/15, Broekman Institute Ltd., Someren, The Netherlands). Room temperature was 22-23 °C. Light period was from 6.00 h to 20.00 h. Three weeks before the experiment all rats were provided with a silicon heart catheter through the right jugular vein for constant infusion of tracer (Steffens, 1969). Rats were conditioned on a high protein (HP) diet (210 g casein/kg feed) or a low protein (LP) diet (75 g casein/kg feed) from 7 weeks of age. Composition of diets was reported earlier

(Schreurs *et al.* 1992). From 2 wks before the experiment feeding was restricted to the period between 9.00 h and 18.00 h.

$^{14}CO_2$ Breath test

Overnight fasted growing rats were subjected to three 8 h constant infusions of labeled leucine. Tracers, L-[1-^{14}C]-leucine (carboxyl, CL) and L-[U-^{14}C]-leucine (universal, UL) (Amersham, Den Bosch, The Netherlands), were diluted with deionized water and infused at a rate of 250 ul/h. First and second, rats were infused with CL (4.6 kBq/h; 2.0 GBq/mmol) with a 2 d interval. Third, rats were infused with UL (4.6 kBq/h; 11.4 GBq/mmol). Two h after start of the first infusion rats were either offered a 5 g HP or LP meal for 30 min. The same rats were offered the other of two meals (HP, LP) at the second test. For the third test (UL) only one of two meals (HP, LP) was offered.

Mature rats were subjected to two constant infusions of labeled leucine. The same tracers were used as in growing rats. First, rats were infused with CL for 8 h (4.6 kBq/h) and second with UL for 7 h (21.1 kBq/h). Three hours after the start of both infusions rats were offered a HP or LP meal.

A small number of mature rats was offered a protein free meal. CL and UL measurements were not always performed in the same rat.

During the experiment the rat was kept in a perspex cage (25x25x15 cm), which was ventilated at a rate of 1.5 l/min. Expired CO_2 was trapped in 250 ml KOH solution (0.3 mol/l). Traps were refreshed per h and 20 ml samples were taken at 15 or 30 min intervals. A 10 ml sample was mixed with 10 ml liquid scintillation cocktail (Insta-Gel, Packard, Groningen, The Netherlands). Expired radioactivity was determined using a liquid scintillation analyser (TRI-CARB 1900 CA, Packard, Groningen, The Netherlands) and expressed as percentage of the dose infused during the time interval of the sample (modified for constant infusion from Schreurs *et al.* 1992).

Statistical analysis

Group means were analysed with Student's *t* test (SPSS Inc., 1988). The data per hour in **Table 2 and 3** were calculated as average of 2 or 4 samples. These data were subjected to analysis of variance (ANOVA); data for protein free meals were excluded from ANOVA. For growing rats the CL data for the HP and LP meal were obtained from the same rat, therefore the 'meal effect' was nested within the 'diet effect'. The differences between growing and mature rats were than investigated with Student's *t* test for both the HP meal and the LP meal.

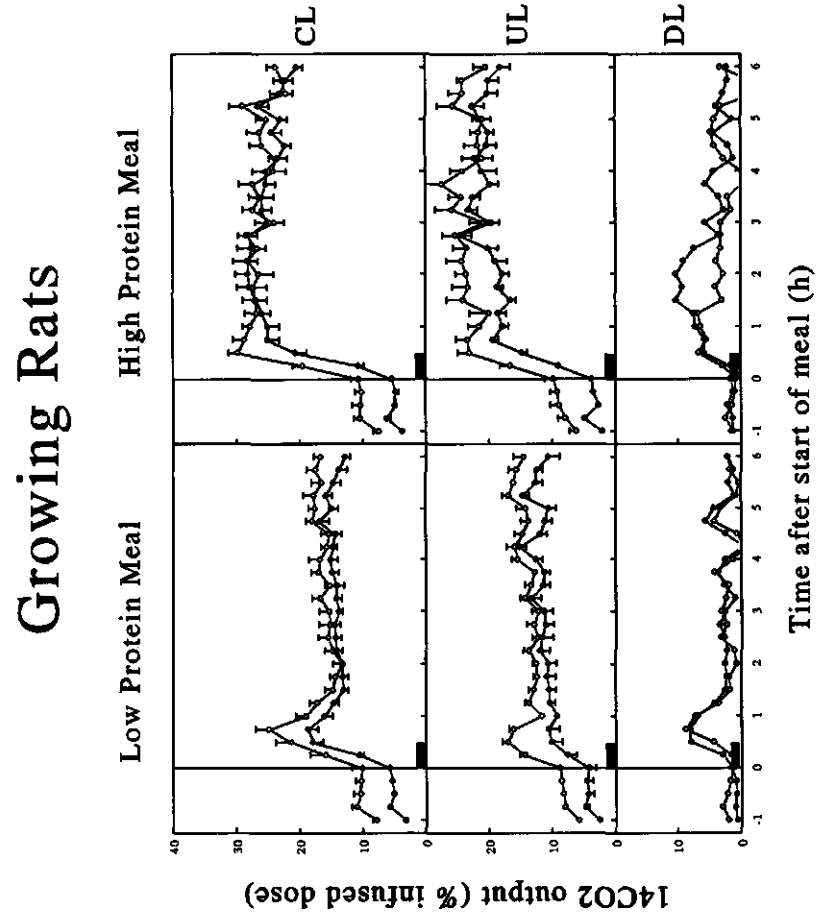
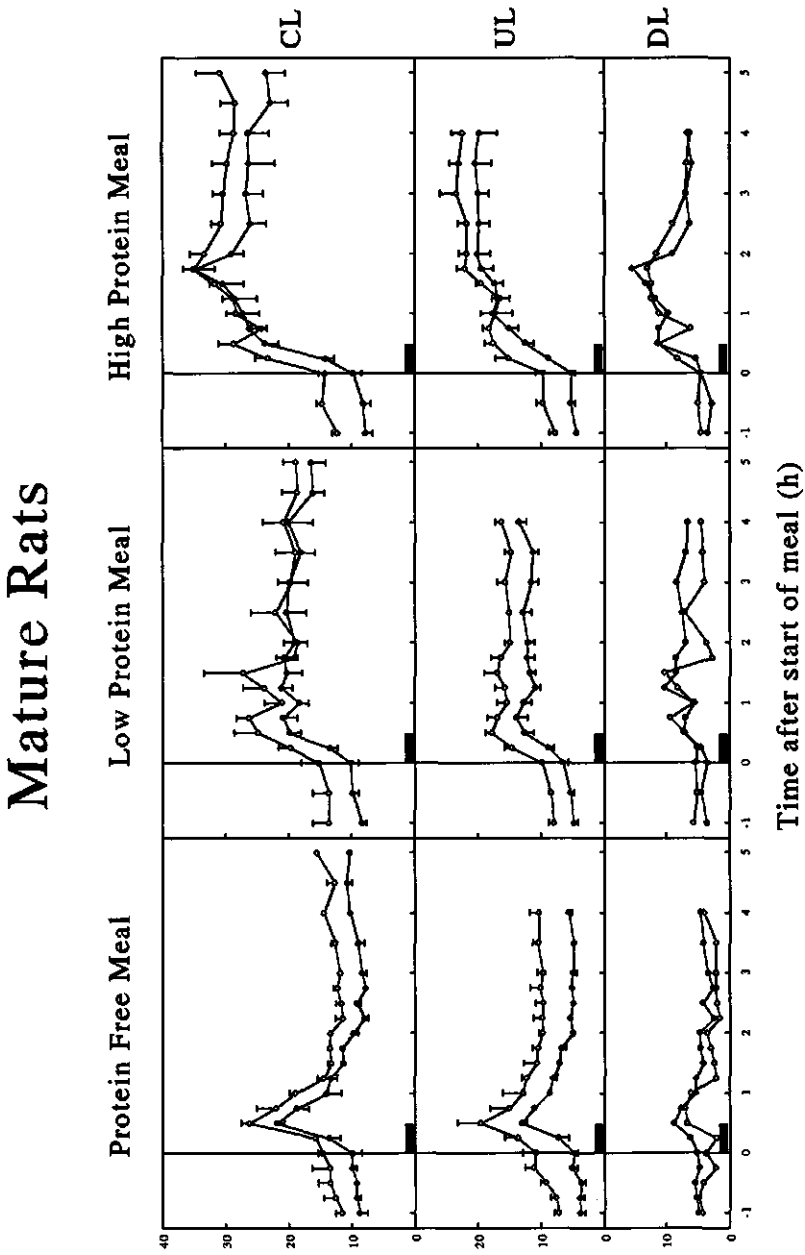


Figure 1. Meal-induced changes in $^{14}CO_2$ output. The test meal was offered from 0 to 0.5 h. Vertical bars indicate SE. Every panel shows two $^{14}CO_2$ output curves, for rats conditioned on the high protein diet (open circles) and the low protein diet (filled circles). CL, carboxyl labeled leucine; UL, universally labeled leucine; DL, difference between CL and UL.


Mature Rats
Protein Free Meal
Low Protein Meal
High Protein Meal
CL
UL
DL
14CO2 output (% infused dose)
Time after start of meal (h)

RESULTS

Body weight and feed intake

Table 1 shows the differences in body weight as a result of the level of chronic protein supply for growing and mature rats. Feed intake (g.kg body weight^{-1}.d^{-1}) was not different between diets.

Table 1.
Body weight.

Diet...	High protein	Low protein
	Mean ± SE	Mean ± SE
Growing rats	187 ± 12	138 ± 5#
Mature rats	426 ± 11	336 ± 11#

Significantly different from high protein diet (P<0.001).

$^{14}CO_2$ Breath test

Figure 1 shows the meal-induced changes in $^{14}CO_2$ output for all groups. In the overnight fasted state (*before* the meal) $^{14}CO_2$ output was lower for the LP *v.* HP diet (P<0.001) and lower for the growing *v.* mature rats (P<0.001). CL and UL data show largely the same differences in the overnight fasted state (**Table 2 and 3**).

Both CL and UL data in **Figure 1** show the fast increase in $^{14}CO_2$ output *during* ingestion of the meal, although the increase is more pronounced for the CL data.

After a protein free meal $^{14}CO_2$ output decreased to the fasted level, with the same difference between the LP and HP diet (P<0.05). The transient nature of this increase indicated the *first response* to the meal. This could not be observed in all figures because of the overlap with the *second response* of $^{14}CO_2$ output to the meal. The second response of $^{14}CO_2$ output to the meal was determined by the protein content of the meal (**Table 2**). After the LP meal $^{14}CO_2$ output was higher compared to the protein free meal (P<0.05), and after the HP meal it was higher compared to the LP meal (P<0.05).

After the meal the effect of diet, as observed in the fasted state, disappeared for the CL data but not for the UL data. The CL data were lower for growing *v.* mature rats, after the HP meal in hour 2 (P<0.05) and after the LP meal in hour 2, 3 and 4

($P<0.005$, $P<0.05$ and $P<0.1$ respectively). The UL data did not show differences between growing and mature rats.

The bottom row of **Figure 1** shows the difference in $^{14}CO_2$ output between CL and UL curves. This difference increases transiently after ingestion of a meal.

DISCUSSION

Introduction

Several studies have investigated the effect of feeding on metabolic leucine utilisation in humans (Garlick *et al.* 1980; Motil *et al.* 1981; Rennie *et al.* 1982; Clugston & Garlick, 1982; Hoffer *et al.* 1985; Melville *et al.* 1989). In these studies the specific activity of plasma leucine (or ketoisocaproate) and breath CO_2 was measured in the *steady state*, in order to calculate leucine flux and leucine oxidation. To our knowledge no attempts have been made to investigate *non-steady state leucine kinetics*, which is more realistic and appreciates physiological limits in the capacity to use dietary protein. In the present study we have developed a model to investigate the non-steady state. At present we accept the limitations of the model and compare meal-induced changes in metabolic leucine utilisation between different physiological conditions.

The model

During constant infusion of labeled leucine the amount of expired labeled carbon dioxide has been measured. This provides a value for the percentage of labeled leucine that is oxidized. In the case of carboxyl labeled leucine this percentage constitutes the part of labeled leucine that is subjected to decarboxylation, the first irreversable step in the degradation of this amino acid. The percentage decarboxylation indicates which part of the labeled leucine pool is not used for protein synthesis. In this way *metabolic amino acid utilisation* can be studied in a non-steady state, e.g. during the transition from the fasted to the fed state. However, changes in $^{14}CO_2$ output can be the result of 1. changes in the percentage of leucine subjected to decarboxylation or total oxidation, but also of 2. changes in $^{14}CO_2$ recovery, and 3. changes in the free leucine pool size and thus in the leucine specific activity. The second and third cause might either overestimate or underestimate the real percentage of decarboxylation or total oxidation. Therefore $^{14}CO_2$ output is not a direct measure of the percentage of leucine subjected to decarboxylation (CL) or total oxidation (UL), but indicates changes in metabolic leucine utilisation.

Table 2.
Effects of acute (meal) and chronic (diet) protein supply on $^{14}CO_2$ output in growing and mature rats, investigated with constant infusion of L-[1-^{14}C]-leucine.

Diet...	High protein		Low protein		ANOVA (P)[1]	
Meal...	High protein	Low protein	High protein	Low protein	Diet	Meal
Time	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE		
			Growing rats			
	(n=7)	(n=7)	(n=11)	(n=11)		
0	10.5 ± 1.0	10.5 ± 0.8	5.4 ± 0.4	5.5 ± 0.2	0.001	NS
1	26.1 ± 1.3	20.3 ± 1.7	20.1 ± 1.4	15.8 ± 0.9	0.001	0.005
2	26.9 ± 1.7	15.0 ± 1.0	27.4 ± 1.6	13.5 ± 0.7	NS	0.001
3	26.8 ± 1.2	15.1 ± 1.1	27.1 ± 1.7	14.3 ± 0.8	NS	0.001
4	26.5 ± 1.5	16.5 ± 1.1	25.6 ± 1.3	14.6 ± 1.0	NS	0.001
5	25.2 ± 1.2	16.7 ± 0.8	23.3 ± 0.8	15.3 ± 1.0	NS	0.001
6	24.4 ± 1.2	17.2 ± 1.2	22.6 ± 1.1	14.4 ± 0.9	0.05	0.001
			Mature rats			
	(n=7)	(n=5)	(n=8)	(n=5)		
0	14.6 ± 0.9	14.5 ± 2.5	9.1 ± 1.3	10.1 ± 1.1	0.002	NS
1	26.2 ± 1.5	23.1 ± 2.4	22.9 ± 2.1	18.2 ± 1.4	0.049	0.052
2	32.2 ± 1.3	22.3 ± 3.2	30.7 ± 2.7	20.4 ± 2.1	NS	0.001
3	30.6 ± 1.4	21.0 ± 2.8	26.5 ± 2.6	20.2 ± 3.0	NS	0.004
4	29.3 ± 1.9	20.0 ± 3.0	26.4 ± 3.6	19.2 ± 3.1	NS	0.022
5	29.7 ± 2.8	19.5 ± 2.1	23.3 ± 2.9	16.4 ± 2.0	0.078	0.006

[1] Analysis of variance of growing rats: meal effect nested within diet effect.

Table 3.
Effects of acute (meal) and chronic (diet) protein supply on $^{14}CO_2$ output in growing and mature rats, investigated with constant infusion of L-[U-^{14}C]-leucine.

Diet...	High protein		Low protein		ANOVA (P)[1]	
Meal...	High protein	Low protein	High protein	Low protein	Diet	Meal
Time	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE		
			Growing rats			
	(n=7)	(n=7)	(n=7)	(n=5)		
0	9.0 ± 1.1	8.4 ± 0.3	3.8 ± 0.1	4.5 ± 0.7	0.001	NS
1	21.1 ± 1.6	14.8 ± 0.6	15.2 ± 0.5	9.4 ± 1.2	0.001	0.001
2	22.8 ± 2.2	13.0 ± 0.5	17.9 ± 1.0	10.7 ± 1.0	0.014	0.001
3	23.5 ± 2.0	12.8 ± 0.7	20.9 ± 1.5	11.5 ± 1.4	NS	0.001
4	25.4 ± 1.9	14.1 ± 0.6	21.4 ± 1.2	12.2 ± 1.0	0.030	0.001
5	21.3 ± 1.2	14.7 ± 1.1	20.9 ± 1.1	12.3 ± 0.8	NS	0.001
6	24.8 ± 1.8	15.9 ± 0.6	20.3 ± 1.0	12.7 ± 1.0	0.005	0.001
			Mature rats			
	(n=6)	(n=8)	(n=6)	(n=8)		
0	9.8 ± 1.0	9.2 ± 0.5	5.4 ± 0.7	6.0 ± 0.7	0.001	NS
1	17.1 ± 1.2	16.2 ± 1.1	13.5 ± 1.5	12.1 ± 1.3	0.005	NS
2	20.0 ± 0.8	16.1 ± 1.3	18.4 ± 1.6	11.9 ± 0.8	0.017	0.001
3	22.6 ± 1.7	15.4 ± 0.8	19.9 ± 1.7	12.2 ± 1.1	0.028	0.001
4	22.8 ± 1.0	15.6 ± 0.9	20.1 ± 2.8	12.3 ± 1.0	0.033	0.001

[1] Significance of diet and meal effect by analysis of variance (P<0.05).

Fasting

The level of chronic protein supply and stage of development of the animal determine metabolic leucine utilisation after an overnight fast. If labeled leucine is not used differently from unlabeled leucine, this indicates a higher reutilisation of endogenous leucine for the LP diet compared to the HP diet. This is in line with the general idea

that adaptation to a LP diet involves a more efficient use of IAAs for protein synthesis (Harper, 1986; Young & Marchini, 1990). It is also in line with a lower amplitude in diurnal cycling of protein (Millward & Rivers, 1988), since amino acid losses are reduced after an overnight fast. This increase in reutilisation of leucine on a LP diet was observed in both growing and mature animals, while growth resulted in an extra increase in reutilisation.

Feeding

The effect of meal ingestion on $^{14}CO_2$ output can be devided in two parts. The first response (upto 1.5 h) originates from metabolic changes that are related to meal ingestion and/or carbohydrate feeding. Meal ingestion might result in a general 'stress' response that is not specific for amino acid metabolism. Carbohydrate feeding has been shown to result in a decreased endogenous leucine influx (protein breakdown) (Nissen & Haymond, 1986; Nair *et al.* 1987; Pacy *et al.* 1991), which acutely reduces the free leucine pool size and increases the specific activity. Especially the latter consideration reduces the validity of the measurement, but it appears from the results that this is only transient.

The second response (after 1.5 h) is determined by the protein content of the meal. This indicates that acute dietary amino acid influx into the metabolic pool overrules the effect of endogenous amino acid influx (from protein breakdown) on metabolic leucine utilisation. This is in line with the observation that protein breakdown is decreased upon feeding (Garlick *et al.* 1990). Feeding repeated small meals has been observed to increase the percentage of leucine flux subjected to oxidation in a steady state by about 1.5 times (Garlick *et al.* 1980; Motil *et al.* 1981; Rennie *et al.* 1982; Clugston & Garlick, 1982; Hoffer *et al.* 1985; Melville *et al.* 1989). Intermittent meal ingestion is shown to increase the percentage of labeled leucine as much as 6 times, depending on the physiological-nutritional condition before the meal and the protein content of the meal.

In order to stress the importance, three comments on this observation. First, the overruling effect of acute supply is absent after ingestion of a protein free meal. The above mentioned effect of carbohydrate feeding provides a mechanism to reduce endogenous leucine losses after a protein free meal, but also a mechanism to gain protein after a LP or HP meal. Second, the overruling effect of acute supply is observed both after a LP and a HP meal. Although growth is restricted on a LP diet, a LP meal increases the percentage of labeled leucine that is subjected to decarboxylation. This indicates that even a meal with a marginal protein level negatively affects metabolic leucine utilisation and thus suggests relative ineffective

utilisation of the supply of leucine. Third, the percentage of labeled leucine subjected to decarboxylation is lower in the growing animals compared to the mature animals, especially after a LP meal. This indicates that especially the LP meal can be used more efficiently by growing animals. This might be related to the higher fractional protein synthesis rate in growing *v.* mature animals (Waterlow *et al.* 1978).

It is well recognized that during adaptation to a different dietary protein level the N-balance shows a delayed response (Munro, 1964). The present study indicates that postprandial amino acid utilisation is mainly affected by acute protein supply, with minor influences of the physiological-nutritional condition of the animal to the use of dietary protein. This suggests that the delay in response is due to subsequent postabsorptive amino acid utilisation. And is in line with the observation that an acute change to a LP diet reduces protein gain but not subsequent protein loss (Millward *et al.* 1990).

Potential of breath test

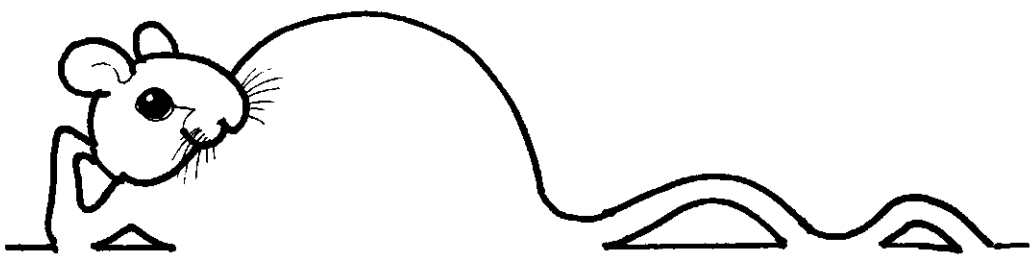
We suggested earlier (Schreurs *et al.* 1992) that the combined use of CL and UL in a breath test has potential value in evaluating relative oversupply of an amino acid.

During transition from the fasted to the fed state the difference between CL and UL increases transiently. This increase in $^{14}CO_2$ output is more apparent in CL than in UL measurements, and might be related to above mentioned aspects of the first response to a meal. The second response to a meal (between 1.5 h and 2.5 h) shows an increase in the difference between CL and UL after a HP meal in three cases: growing rats/LP diet, mature rats/LP diet, and mature rats/HP diet. This indicates that in these animals leucine supply with a HP meal is high relative to the ability of the animals to handle it. This is in line with our previous suggestion (Schreurs *et al.* 1992).

Conclusion

The present study indicates that 1. metabolic leucine utilisation after overnight fasting depends on the level of chronic protein supply and stage of development of the animal, 2. metabolic leucine utilisation after feeding depends primarily on the level of acute protein supply, 3. the transient increase in non-protein label retention suggests a temporal oversupply of leucine relative to the actual metabolic state.

Since feeding, especially intermittent feeding, and changes in dietary protein level have a major influence on metabolic leucine utilisation, it is likely that dietary protein and IAA requirements change with dietary habits.



Chapter 2

EFFECTS OF FASTING AND FEEDING ON METABOLIC LEUCINE UTILISATION IN GROWING RATS

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Effects of fasting and feeding on metabolic leucine utilisation were investigated in growing rats. Rats were conditioned on a high protein (HP) diet (210 g casein/kg feed) or a low protein (LP) diet (75 g casein/kg feed). After an overnight (18 h) or prolonged (30 h) fast rats were offered a 5 g HP, LP or protein free (PF) meal for 30 min. Rats were subjected to an 8 h $^{14}CO_2$ breath test, during a constant intravenous infusion or oral feeding of L-[1-^{14}C]-leucine (CL) or L-[U-^{14}C]-leucine (UL). Expired $^{14}CO_2$ was expressed as percentage of infused or consumed dose. With intravenous tracer infusion in the fasted state protein restriction decreased ($P<0.001$) and prolonged fasting increased ($P<0.001$) $^{14}CO_2$ output. Refeeding 30 h fasted LP rats with a PF meal decreased $^{14}CO_2$ output to the 18 h fasted level, but not in 30 h fasted HP rats. Refeeding with a HP meal resulted in the same $^{14}CO_2$ output, independent of previous protein restriction or fasting duration. Not untill 6 h after the HP meal 30 h fasting resulted in a higher $^{14}CO_2$ output ($P<0.05$). With oral tracer administration refeeding with a HP meal resulted in a slight decrease in $^{14}CO_2$ output with protein restriction ($P<0.001$) but a profound increase with fasting duration ($P<0.001$). In conclusion, 1. the protein level of feeding determines the level of leucine losses, and 2. longer than overnight fasting may profoundly decrease the efficiency of utilisation of endogenous but primarily dietary leucine.

Amino acid metabolism: Leucine: Dietary protein: Fasting: Rat

INTRODUCTION

In the overnight fasted state leucine oxidation is lower compared to the fed state (Garlick *et al.* 1980; Motil *et al.* 1981; Rennie *et al.* 1982; Clugston & Garlick, 1982; Hoffer *et al.* 1985; Melville *et al.* 1989). Fasting however has also been reported to increase leucine oxidation, both in rats (Sketcher *et al.* 1974; Vazquez *et al.* 1986) and in humans (Tsalikian *et al.* 1984; Nair *et al.* 1987; Jensen *et al.* 1988; Knapik *et al.* 1991). This indicates that overnight fasting initially decreases leucine oxidation, but prolonged (beyond overnight) fasting increases leucine oxidation.

Protein restriction has been shown to decrease leucine oxidation (McFarlane & von Holt, 1969; Neale, 1971; Sketcher *et al.* 1974; Waterlow *et al.* 1978; Schreurs *et al.* 1992). Thus leucine oxidation in the fasted state is decreased by protein restriction and increased by prolonged fasting. The effect of these physiological conditions on amino acid utilisation in the postprandial state (after feeding) is not known. The first pass of dietary branched-chain amino acids through the splanchnic tissues has been reported to cause substantial losses (Cortiella *et al.* 1988; Imura *et al.* 1988; Istfan *et al.* 1988; Hoerr *et al.* 1991). This first pass effect is presently investigated with intravenous and oral tracer administration.

In the present study both protein restriction and prolonged fasting have been studied in the postabsorptive (fasted) state and postprandial (fed) state.

MATERIALS AND METHODS

Animals and diets

Growing (10 wks) male Wistar-WU rats (Center for Small Laboratory Animals, Wageningen Agricultural University, Wageningen, The Netherlands) were housed in individual cages with bedding material (Woody-Clean type 8/15, Broekman Institute Ltd., Someren, The Netherlands). Room temperature was 22-23°C. Light period was from 6.00 h - 20.00 h. Rats were provided with a silicon heart catheter through the right jugular vein for constant infusion of tracer (Steffens, 1969), when necessary. Rats were conditioned on a high protein (HP) diet (210 g casein/kg feed) or a low protein (LP) diet (75 g casein/kg feed) for 3 weeks. Detailed composition of diets was reported earlier (Schreurs *et al.* 1992). Rats investigated after an overnight (18 h) fast were fed between 9.00 h and 18.00 h for 2 weeks. Other rats were fed ad libitum and fasted for 6 h or 30 h fasting.

$^{14}CO_2$ Breath test

Rats were subjected to an 8 h $^{14}CO_2$ breath test, either during intravenous constant infusion of tracer (**Experiment 1**) or after oral feeding of a meal mixed with tracer (**Experiment 2**). Two tracers were used, first L-[1-^{14}C]-leucine (carboxyl, CL; 2.0 GBq/mmol) and second L-[U-^{14}C]-leucine (universal, UL; 11.4 GBq/mmol; Amersham, Den Bosch, The Netherlands).

Experiment 1. Fasted rats (6 h, 18 h and 30 h) were subjected to 8 h constant intravenous infusions of CL or UL (4.6 kBq/h; 250 μl/h). After 2 h of infusion 18 h and 30 h fasted rats were offered a HP meal, a LP meal (only 18 h fasted rats) or a protein free (PF) meal (only 30 h fasted rats).

Experiment 2. Fasted rats (18 h and 30 h) were fed a HP meal (5 g; 30 min), mixed with CL (17 kBq; 0.5 ml deionized water).

During the breath test rats were kept in perspex cages, ventilated at a rate of 1.5 l/min. Expired CO_2 was trapped in a KOH solution, samples were analysed for radioactivity and $^{14}CO_2$ output was expressed as percentage of the infused or consumed dose.

Statistical analysis

Data were analysed for the effect of protein restriction and prolonged fasting by ANOVA (SPSS Inc., 1988). Differences between group means were analysed by Student's *t* test.

RESULTS

Experiment 1

Figure 1 shows the effect of fasting on $^{14}CO_2$ output at plateau during intravenous tracer infusion. Protein restriction reduced $^{14}CO_2$ output both in the fed (0 h fasting) and fasted state ($P<0.001$). Fasting for 18 h reduced $^{14}CO_2$ output below the level of 6 h fasting ($P<0.05$), while 30 h fasting increased $^{14}CO_2$ output significantly for the LP diet ($P<0.001$).

Table 1 shows the effect of protein restriction (diet) and prolonged fasting (fast) on $^{14}CO_2$ output. For CL both diet and fasting had a significant effect on $^{14}CO_2$ output in the overnight fasted state. Refeeding with a HP meal increased $^{14}CO_2$ output dramatically, whereas the effects of diet and fasting disappeared. But 6 h after refeeding fasted rats with a HP meal the effect of fasting returned ($P<0.05$), and resulted in a higher $^{14}CO_2$ output for 30 h *v.* 18 h fasted rats. For UL the diet effect

remained significant after refeeding with a HP meal, while the fasting effect was not significant. Contrary to the CL values, the UL values were not different between the 18 h and 30 h fasted group.

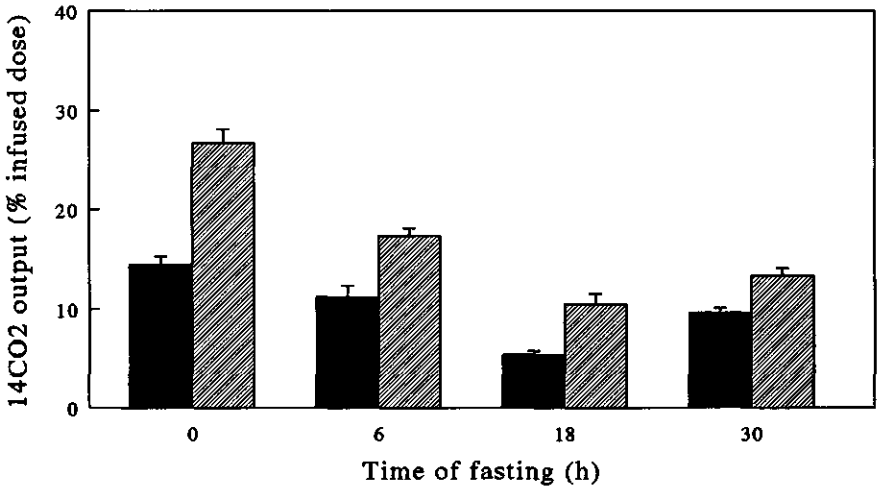


Figure 1. Effect of fasting on $^{14}CO_2$ output during intravenous infusion of tracer. Low protein group (black bars) and high protein group (dashed bars). Hour 0, just after a meal; hour 6, 6 h fasted; hour 18, overnight fasted; hour 30, 30 h fasted.

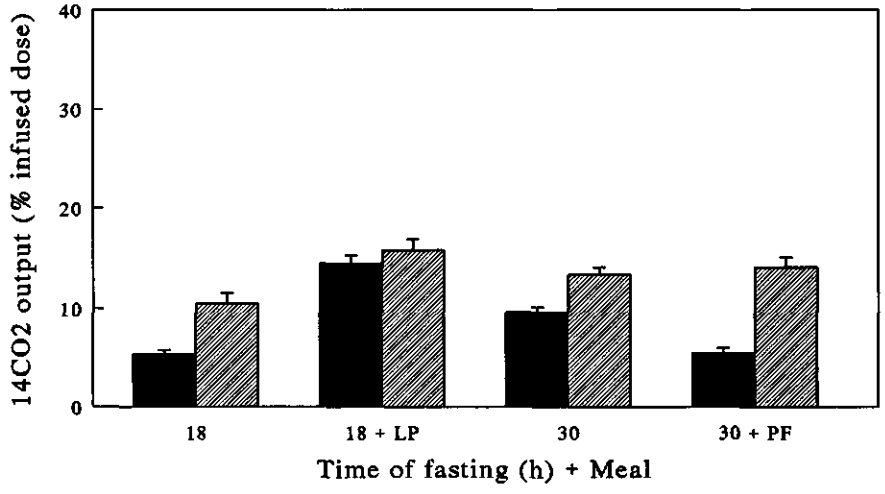


Figure 2. Comparison of the 18 h and 30 h fasted $^{14}CO_2$ output level with the level after feeding a low protein (LP) meal or a protein free (PF) meal. For the PF meal the mean of 4 values between 2.5 h and 3.25 h after the meal was calculated per animal. Low protein group (black bars) and high protein group (dashed bars).

Figure 2 shows the effect of feeding on $^{14}CO_2$ output. Refeeding 30 h fasted LP rats with a PF meal caused $^{14}CO_2$ output to decrease significantly ($P<0.005$) to the 18 h fasted level, but not for HP rats. Refeeding 18 h fasted rats with a LP meal caused $^{14}CO_2$ output for both diets to increase to the same level.

Figure 3 shows the results after feeding a HP meal. With i.v. tracer administration the level of $^{14}CO_2$ output at 4 h after the HP meal was not different between diet and fasting groups. **Table 1** shows that $^{14}CO_2$ output at 6 h after the meal tended to decrease for the 18 h fasted group, but not for the 30 h fasted group.

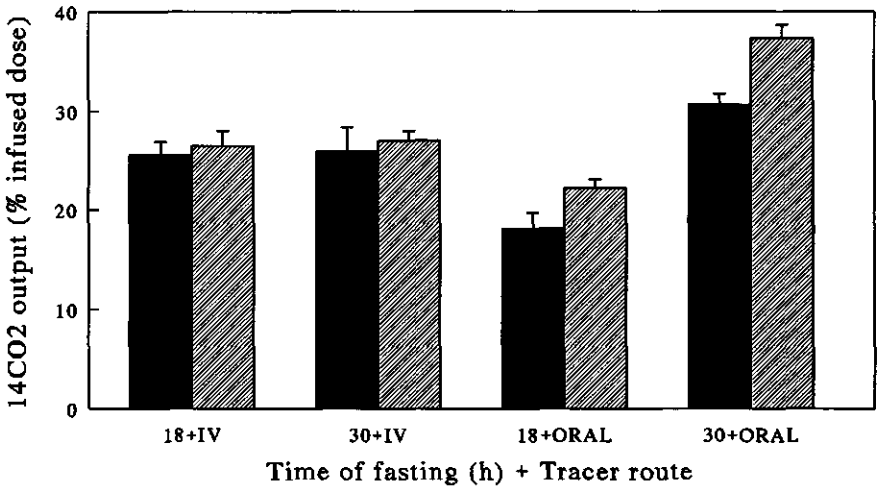


Figure 3. Interaction between the effect of fasting and tracer administration. Either 18 h or 30 h fasted rats were offered a 5 g high protein meal. I.V., during intravenous infusion of tracer; ORAL, after administration of tracer with the meal. Low protein group (black bars) and high protein group (dashed bars).

Experiment 2

Table 1 shows that with oral tracer administration the level of $^{14}CO_2$ output was highly dependent upon diet ($P<0.001$) and fasting ($P<0.001$). In the 18 h fasted rats $^{14}CO_2$ output seemed to be lower with oral *v.* i.v. tracer administration, but for the 30 h fasted rats $^{14}CO_2$ output was much higher after oral tracer administration. The difference between the 18 h and 30 h fasted rats initialised immediately after the meal (data not shown). While the difference between the HP and LP diet appeared 3.5 h after the meal in the 18 h fasted rats, but 1.5 h after the meal in the 30 h fasted rats (data not shown).

Table 1.
Effects of diet and fasting on postabsorptive and postprandial $^{14}CO_2$ output during intravenous and after oral tracer administration.

Diet...	High protein		Low protein		ANOVA (P)[1]	
Fast...	18 h	30 h	18 h	30 h	Diet	Fast
Time[2]	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE		
Intravenous tracer administration / L-[1-^{14}C]-leucine						
	(n=7)	(n=7)	(n=11)	(n=4)		
0	10.5 ± 1.0	13.0 ± 0.8	5.4 ± 0.4	8.8 ± 0.6	0.001	0.001
2	26.9 ± 1.7	29.3 ± 1.2	27.4 ± 1.6	26.7 ± 4.2	NS	NS
4	26.5 ± 1.5	27.0 ± 1.0	25.6 ± 1.3	25.9 ± 2.5	NS	NS
6	24.4 ± 1.2	26.7 ± 0.4	22.6 ± 1.1	26.0 ± 2.1	NS	0.05
Intravenous tracer administration / L-[U-^{14}C]-leucine						
	(n=7)	(n=5)	(n=7)	(n=5)		
0	9.0 ± 1.1	8.9 ± 0.5	3.8 ± 0.1	5.2 ± 0.3	0.001	NS
2	22.8 ± 2.2	22.1 ± 1.2	17.9 ± 1.0	18.5 ± 1.3	0.05	NS
4	25.4 ± 1.9	23.4 ± 1.4	21.4 ± 1.2	19.5 ± 1.8	0.05	NS
6	24.8 ± 1.8	22.0 ± 1.4	20.3 ± 1.0	20.9 ± 1.3	0.05	NS
Oral tracer administration / L-[1-^{14}C]-leucine						
	(n=5)	(n=4)	(n=4)	(n=7)		
PP[3]	22.2 ± 0.9	37.3 ± 1.4	18.1 ± 1.6	30.7 ± 1.1	0.001	0.001

[1] Significant effect of diet and fast by analysis of variance (P<0.05). [2] At 0 h a HP meal is provided for 30 min; [3] PP, cumulative percentage over postprandial period.

DISCUSSION

Effect of fasting

Leucine oxidation has been shown to depend on the nutritional condition of the animal, with a high rate of leucine oxidation in the fed state. In the overnight fasted state leucine oxidation is lower than in the fed state. Several studies found leucine oxidation to be increased after 2 or 3 days of fasting. One study indicated that both leucine oxidation and leucine flux increased already after 30 h fasting (Tsalikian *et al.* 1984). The same has been reported for the leucine flux. The percentage of leucine flux subjected to leucine oxidation provides information about the inefficiency of leucine utilisation. The results indicate that the percentage leucine oxidation profoundly decreases with fasting, upto the 'largest' interprandial period (most commonly the overnight fasted state). For both the abundant HP diet and the marginal LP diet the efficiency of reutilisation increased upto the overnight fasted state. Thereafter the percentage leucine oxidation increased again, especially in the LP group. Since a protein free meal (essentially carbohydrates) reduced this inefficiency from the 30 h fasted to the 18 h fasted level, the cause is probably the lack of energy or more specificly glucose. Although the rise in the percentage leucine oxidation with 30 h fasting was just significant in the HP group, the difference was small and not reversable with a protein free meal.

Several reasons for an increase in leucine oxidation

Net breakdown of muscle protein provides amino acids for both energy metabolism and biosynthetic processes. There is a net efflux of glutamine and alanine from the muscle tissue (Felig, 1975). Glutamine is used by the gut and kidney, and alanine is mainly used for gluconeogenesis in the liver. The proportion of glutamine and alanine in the amino acids released from the muscle is far in excess of the proportion of these amino acids in muscle protein. Most amino acids are metabolised in the liver, but for the branched-chain amino acids (BCAA) the transaminase (BCAAT) activity is high in muscle (Bender, 1985; Hutson, 1989). Therefore the production of both glutamine and alanine seems to depend on the level of BCAAT activity. The product of leucine deamination, α-ketoisocaproate (α-KIC), can then be released and taken up by the liver (rat, Livesey & Lund, 1980) or decarboxylated in muscle tissue (human, Elia & Livesey, 1983). After a 24 h fast it has been demonstrated that the canine liver converts leucine carbon into ketone carbon, which accounted for 10-15% of hepatic ketogenesis (Abumrad *et al.* 1989). Thus there might be a specific requirement for leucine N and C in the 30 h fasted state.

The production of glutamine consumes both glutamate, taken up by muscle (Elia *et al.*, 1989), and α-ketoglutarate (α-KG), a citric acid cycle (CAC) intermediate. Production of alanine in the fasted state consumes pyruvate which is not derived from glycolysis but from oxaloacetate (by the action of phosphoenolpyruvate carboxykinase) and malate (malic enzyme), both CAC intermediates (Snell, 1980). Muscle is mainly consuming free fatty acids (FFA) for energy production, with an increasing participation of ketones (Cahill & Aoki, 1980). But for oxidation of acetyl-CoA in the CAC the intermediates have to be present in sufficient amounts. Both valine and isoleucine supply succinyl-CoA to the CAC under fasting conditions. Leucine however only supplies ketones (acetyl-CoA and acetoacetate), but is catabolysed by the same BCAAT and branched-chain keto acid dehydrogenase (BCKA-DH) as valine and isoleucine (Bender, 1985).

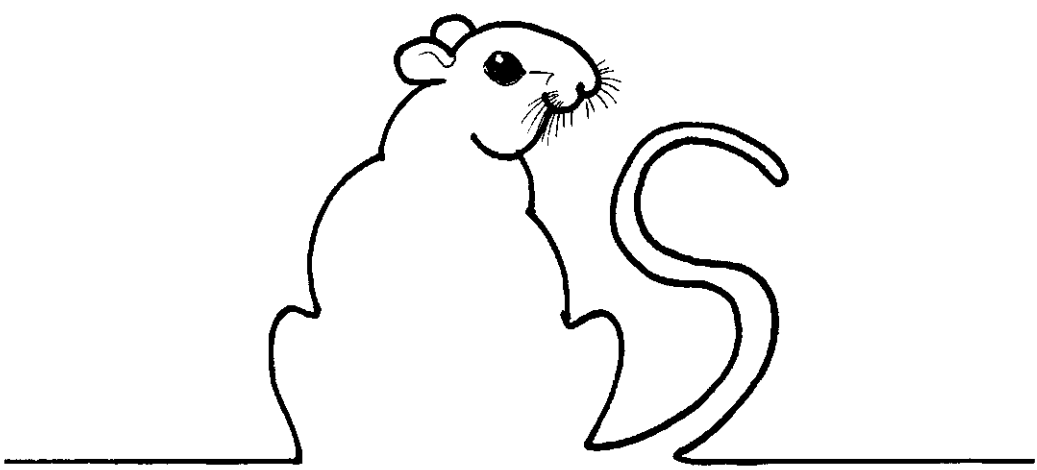
Regulation of leucine oxidation in the muscle might also regulate protein breakdown. BCAA oxidation and the FFA oxidation consume the same cofactors, like coenzyme A (CoA) and nicotinamide adenine dinucleotide (NAD^+), and results in the same end-products, acetyl-CoA and succinyl-CoA (odd number carbon chain). In prolonged starvation (days!) the depletion of cofactors may be the signal to reduce leucine oxidation, and thus protein breakdown. Especially the increased level of ketones results in increased ketone utilisation by the brain, which spares glucose and therefore muscle protein. An in vitro study showed that addition of ketone bodies to muscle homogenate of 4 day fasted rats increased leucine decarboxylation (Paul & Adibi, 1978). Leucine infusion in 24 h fasted dogs resulted in increased ketogenesis and almost complete suppression of liver protein breakdown (Abumrad *et al.* 1989). And in the overnight fasted human it was found that ß-Hydroxybutyrate (ß-HOB) administration decreased leucine oxidation (Nair *et al.* 1988). Thus the fasting induced ketone body production is primarily effective in conservation of leucine and body protein in early starvation (prolonged fasting, roughly between 1 and 2 days). During starvation ketone production from FFA only slowly increases during several days, which might indicate that leucine (or BCAA) have a special function in this period.

Therefore leucine might play a specific role in energy metabolism, especially during fasting. However in the present experiment only the percentage decarboxylation of leucine and not complete oxidation of leucine is increased with fasting. This indicates that leucine is not used as an energy source, but that it provides N for alanine and glutamine. Alanine for gluconeogenesis in the liver and glutamine as an energy substrate for the gut. Both pathways may be decreased upon carbohydrate feeding. Carbohydrate feeding might also increase the ATP concentration (energy charge), which has been demonstrated to decrease the fasting induced increase in BCKA-DH

activity (Paul & Adibi, 1982; Kasperek, 1989).
Leucine may also be decarboxylated because gluconeogenic amino acids have been extracted from the free amino acid pool, which reduces reutilisation of leucine for protein synthesis.

Tracer administration
Since the oral administration of tracer was as a bolus and the intravenous administration as an infusion, we will not compare the absolute values between individual groups. It is clear however that the effects of protein deprivation and fasting were significant with oral, but not with intravenous, administration. This discrepancy indicates differences in metabolic leucine utilisation between the splanchnic and peripheral tissues. With oral administration all the tracer is absorbed via the gut mucosa and transported to the liver, before entering the systemic circulation.
The BCAA have been claimed to escape splanchnic metabolism and are taken up by peripheral tissues (Wahren *et al.* 1976; Elia *et al.* 1989). The ingestion of a HP meal provides a high exogenous leucine influx, which is in part removed on first pass through the splanchnic tissues (Cortiella *et al.* 1988; Istfan *et al.* 1988). BCAAT activity has been demonstrated in several tissues, including liver and gut (Goto *et al.* 1977; Abumrad *et al.* 1989). Also transamination of leucine in the splanchnic tissues and release of α-KIC into the systemic circulation has been found in humans after an overnight fast (Hoerr *et al.* 1991). When transamination of leucine is increased in the gut or liver by fasting, than more α-KIC can be decarboxylated in the liver and not released into the systemic circulation. This would also facilitate protein deprivation, which decreases the liver BCKA-DH activity (Block *et al.* 1990), to regulate splanchnic leucine decarboxylation. Whole-body leucine decarboxylation is primarily determined by the level of acute protein supply (**Chapter 1**). That fasting has a sustained effect on whole-body leucine decarboxylation (intravenous infusion) was shown by the significant effect of fasting at 6 h after a HP meal.

In conclusion, it is shown that prolonged (beyond overnight) fasting increases percental leucine oxidation, which is only in part reversible by ingestion of carbohydrates. Ingestion of a high protein meal increased percental leucine oxidation. With oral tracer administration the condition of the animal had more impact on the leucine oxidation measurement, than with intravenous tracer administration. Protein deprivation and primarily prolonged fasting had a profound effect on splanchnic tissue (possibly gut mucosa), which lasted at least several hours into the postprandial phase.



Chapter 3

EFFECTS OF DIETARY PROTEIN LEVEL AND LEUCINE ENRICHED MEALS ON METABOLIC LEUCINE UTILISATION IN GROWING AND MATURE RATS

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Effects of dietary protein level and leucine enriched meals on metabolic leucine utilisation were investigated in growing and mature rats. Rats were conditioned on a high protein (HP) diet (210 g casein/kg feed) or a low protein (LP) diet (75 g casein/kg feed). Overnight fasted rats were offered a 5 g HP meal for 30 min at the onset of a 7.5 h $^{14}CO_2$ breath test. The meal contained either L-[1-^{14}C]-leucine (CL) or L-[U-^{14}C]-leucine (UL). Meals enriched with synthetic leucine (70 mg leucine/g protein) were used to investigate specific leucine oversupply. General amino acid oversupply was investigated with a 5 g very high protein (VHP) meal (500 g casein/kg feed). The CL data indicated a slightly lower $^{14}CO_2$ output in hour 1 ($P<0.01$) and 2 ($P=0.055$) for growing *v.* mature rats. Initially the hourly $^{14}CO_2$ output was not different between diets, but the eventual cumulative $^{14}CO_2$ output was lower for the LP *v.* HP diet in growing rats ($P<0.05$). The UL data indicated no effect of diet or age. Leucine enriched meals increased hourly $^{14}CO_2$ output for both CL and UL ($P<0.001$). The difference in $^{14}CO_2$ output (CL-UL) was only higher in hour 1 ($P<0.001$), and might be related to a time lag in $^{14}CO_2$ recovery for UL. The VHP meal supplied a similar amount of leucine which resulted in a similar level of $^{14}CO_2$ output, although more delayed. In conclusion the present study indicated that dietary protein level and growth only marginally affect metabolic leucine utilisation after a meal. However, an increase in protein or leucine content of the meal had dramatic negative effects on postprandial metabolic leucine utilisation.

Amino acid metabolism: Leucine: Dietary protein: Growth: Rat

INTRODUCTION

Chronic protein restriction has been demonstrated to decrease the percental leucine oxidation both in the fasted and fed state (McFarlane & von Holt, 1969; Neale, 1971; Sketcher *et al.* 1974; Neale & Waterlow, 1974; Birt & Clark, 1976; Waterlow *et al.* 1978). Metabolic amino acid utilisation has been investigated in the fed state with different feeds and feeding regimes (Brookes *et al.* 1972; Kim *et al.* 1983; Harper & Benjamin, 1984; Ball & Bayley, 1985; Batterham & Bayley, 1989). But no distinction has been made between the effects of the previous dietary protein level (chronic protein supply) and the present dietary protein level (acute protein supply) on metabolic amino acid utilisation after a meal.

In the overnight fasted state metabolic leucine utilisation is determined by the effect of chronic protein supply and growth, but after a meal by the effect of acute protein supply (**Chapter 1**). However, in the early postprandial phase (2-4 h after the meal) metabolic leucine utilisation was significantly improved in growing *v.* mature animals (**Chapter 1**).

In the present study the effects of chronic protein supply and growth on postprandial metabolic leucine utilisation were investigated. Furthermore the effect of an acute oversupply of all amino acids (protein) or a specific amino acid (leucine) was investigated.

MATERIALS AND METHODS

Animals and diets

Growing (10 wks) and mature (15 wks) male Wistar-WU rats (Center for Small Laboratory Animals, Wageningen Agricultural University, Wageningen, The Netherlands) were housed in individual cages with bedding material (Woody-Clean type 8/15, Broekman Institute Ltd., Someren, The Netherlands). Room temperature was 22-23°C. Light period was from 6.00 h to 20.00 h. Rats were conditioned on a high protein (HP) diet (210 g casein/kg feed) or a low protein (LP) diet (75 g casein/kg feed) from 5 wks of age. Composition of diets was reported earlier (Schreurs *et al.* 1992). For 1 wk before the experiment feeding was restricted to the period between 8.30 h and 17.30 h.

$^{14}CO_2$ Breath test

The breath test was performed as previously described (Schreurs *et al.* 1992), except

for the route of tracer administration. Rats were offered a meal with either L-[1-^{14}C]-leucine (CL; 2.0 GBq/mmol) and about 4 days later with L-[U-^{14}C]-leucine (UL; 11.4 GBq/mmol; Amersham, Den Bosch, The Netherlands). The tracer dose (17 kBq) was diluted in 0.5 ml water and carefully mixed with a 5 g HP meal (unless mentioned otherwise). Expired $^{14}CO_2$ was collected for 7.5 h. Samples were taken every 30 min, but are presented as hourly average. The $^{14}CO_2$ output is expressed as percentage of consumed dose.

Experiment 1. Overnight fasted growing and mature rats on either diet were investigated with both CL and UL.

Experiment 2. This experiment was identical to **Experiment 1** except for addition of synthetic leucine to the meal (70 mg leucine/g protein; Degussa AG, Hanau, Germany). The amount of synthetic leucine was similar to the leucine content of 1 g casein.

Experiment 3. Overnight fasted mature rats on a HP diet were offered either a protein free (PF) meal or a very high protein (VHP) meal (500 g casein/kg feed) and were investigated with CL.

Statistical analysis

Data were analysed for the effect of diet, age and synthetic leucine by ANOVA (SPSS Inc., 1988). Differences between group means were analysed by Student's *t* test.

RESULTS

Body weight and feed intake

Table 1 shows that body weight was lower for the LP *v.* HP diet ($P<0.001$) for both growing and mature rats. Weight gain over the last week before the experiment was used as an estimate of daily weight gain, which was lower for the LP *v.* HP diet in growing rats ($P<0.001$) and not significantly different in mature rats. Feed intake was lower for LP *v.* HP diet in g/d, but higher for LP *v.* HP diet in g.kg body weight^{-1}.d^{-1}.

$^{14}CO_2$ Breath test

Figure 1 (Experiment 1) shows that hourly $^{14}CO_2$ output was not different between the HP and LP group, both for CL and UL. In hour 1 ($P<0.01$) and 2 ($P=0.055$) after the meal $^{14}CO_2$ output was lower for growing *v.* mature rats, for CL but not for UL.

Figure 2 (**Experiment 2**) shows that hourly $^{14}CO_2$ output was higher after the meal with *v.* without synthetic leucine, both for CL (at least $P<0.05$) and UL ($P<0.001$). The difference in $^{14}CO_2$ output (CL-UL) was mainly increased in hour 1 after the meal with *v.* without synthetic leucine ($P<0.001$).

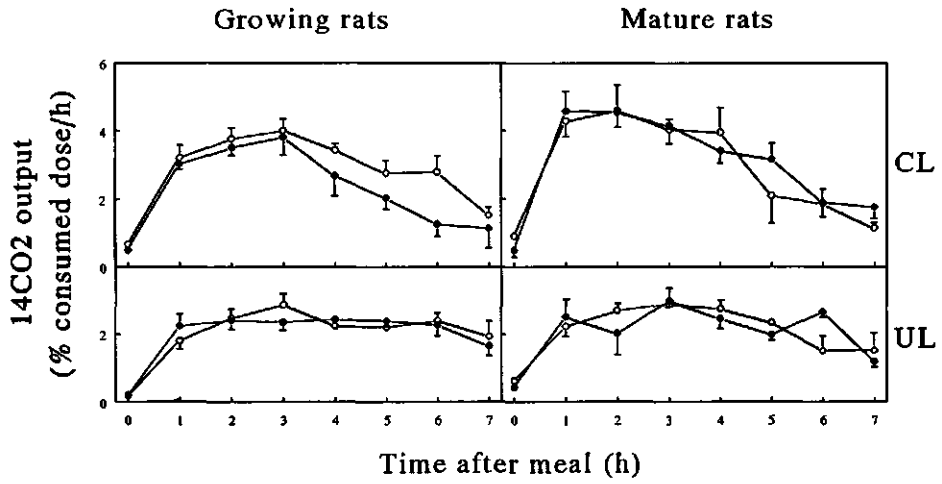


Figure 1. Hourly $^{14}CO_2$ output (% consumed dose/h) after a high protein meal (**Experiment 1**). Every panel shows hourly $^{14}CO_2$ output for rats conditioned on the high protein diet (open circles) and the low protein diet (filled circles). Vertical bars indicate SE.

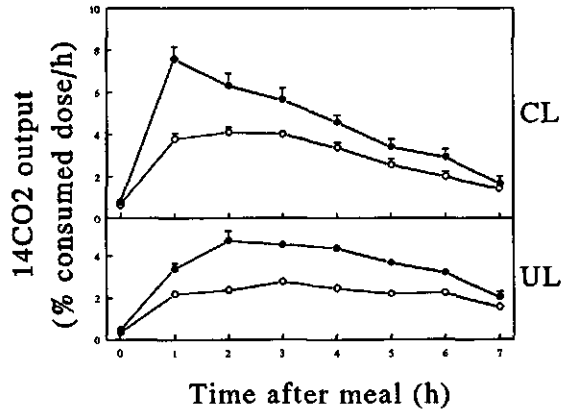


Figure 2. Hourly $^{14}CO_2$ output (% consumed dose/h) after a high protein meal (**Experiment 2**). Every panel shows hourly $^{14}CO_2$ output for meals without synthetic leucine (open circles) and with synthetic leucine (filled circles). Vertical bars indicate SE.

Table 1.
Body weight and weight gain.

Rats...	Growing		Mature	
Diet...	High protein	Low protein	High protein	Low protein
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Body weight (g)	260 ± 8	168 ± 12*	353 ± 13	208 ± 13*
Weight gain (g/d)	5.8 ± 0.3	2.4 ± 0.2*	1.1 ± 0.7	0.9 ± 0.5

* Significantly different from high protein diet ($P<0.001$).

Table 2 (Experiment 1 and 2) shows that the cumulative $^{14}CO_2$ output in growing rats was lower for the LP *v.* HP diet ($P<0.05$), for CL. In general, the cumulative $^{14}CO_2$ output after the meal was not significantly different between the diets and between growing and mature rats, both for CL and UL. For the LP diet the CL data seemed to be lower for growing *v.* mature animals, but this contrast did not reach statistical significance. In all groups the cumulative $^{14}CO_2$ output was higher after the meal with *v.* without synthetic leucine ($P<0.001$).

Figure 3 (Experiment 3) shows that the hourly $^{14}CO_2$ output was higher after a VHP *v.* HP meal ($P<0.001$). Hourly $^{14}CO_2$ output was lower for the PF *v.* HP meal ($P<0.001$).

DISCUSSION

Effect of chronic protein supply

Chronic protein restriction reduced weight gain in growing rats. This was associated with a reduced cumulative percentage of labeled leucine subjected to decarboxylation in growing rats. The hourly percentage leucine oxidation in the early postprandial period (upto 3 h) did not show an effect of diet. This indicates that the response in the early postprandial period largely depends on the protein content of the meal, while the effect of diet may return later. In a previous study with constant tracer infusion (**Chapter 1**) chronic protein restriction improved metabolic leucine utilisation in the overnight fasted (postabsorptive) state. This diet effect disappeared after

ingestion of either a HP or LP meal and returned about 5 hours later.
Since endogenous protein breakdown is decreased upon feeding (Garlick *et al.* 1990), it can be expected that it will increase at a later stage after feeding. The level of endogenous protein breakdown will than depend on the dietary protein level.

Table 2.
Cumulative $^{14}CO_2$ output over 7.5 h.

Rats...	Growing		Mature	
Diet...	High protein	Low protein	High protein	Low protein
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
	Number of observations			
- Leucine	4	5	5	4
+ Leucine	3	5	4	5
	L-[1-^{14}C]-leucine			
- Leucine	22.2 ± 0.9	18.1 ± 1.6#	22.9 ± 2.5	24.0 ± 2.2
+ Leucine[1]	32.8 ± 0.7	28.1 ± 4.0	32.1 ± 2.2	37.9 ± 4.7
	L-[U-^{14}C]-leucine			
- Leucine	16.1 ± 0.4	15.9 ± 0.9	16.5 ± 1.3	16.2 ± 1.3
+ Leucine[1]	27.0 ± 0.5	29.4 ± 0.7#	25.0 ± 0.9	25.7 ± 1.0

Significantly different from high protein diet ($P<0.05$). [1] Addition of synthetic leucine (+ Leucine) resulted in significant higher values in all groups ($P<0.05$).

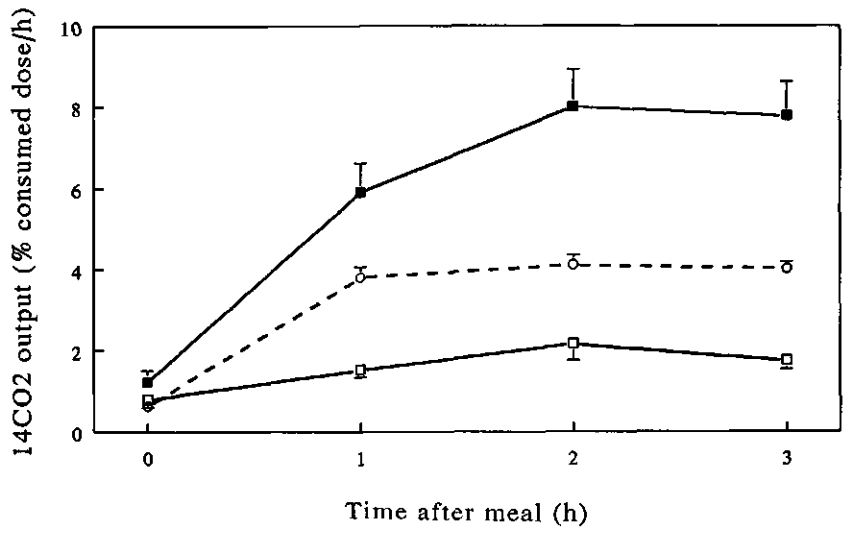


Figure 3. Hourly $^{14}CO_2$ output (% consumed dose/h) after a meal with L-[1-^{14}C]-leucine (**Experiment 3**). Rats conditioned on a high protein diet were offered a protein free meal (open squares) or a very high protein meal (filled squares). The dashed line represents the average of both diets and age groups from **Experiment 1**. Vertical bars indicate SE.

Effect of stage of development

The lower percentage leucine oxidation in the early postprandial period for growing compared to mature rats, indicates an improved initial metabolic leucine utilisation in growing animals. In a previous study with constant tracer infusion (**Chapter 1**) metabolic leucine utilisation was improved in the overnight fasted state in growing compared to mature rats. This effect was still present in the early postprandial period. Diurnal cycling of body protein, which consists of postprandial protein gain and subsequent postabsorptive protein loss (Millward & Rivers, 1988), is relevant to both growing and mature animals. Growth may thus result from either increased protein gain or decreased protein loss, or both. The results indicate that metabolic leucine utilisation is improved in the early postprandial phase. It is likely that this improved utilisation contributes to growth, although neither the extent of this contribution nor the mechanism can be deduced from the present data. Possible mechanisms would be a delayed response of protein breakdown in mature animals, and stimulation of protein synthesis in growing animals.

Effect of synthetic leucine

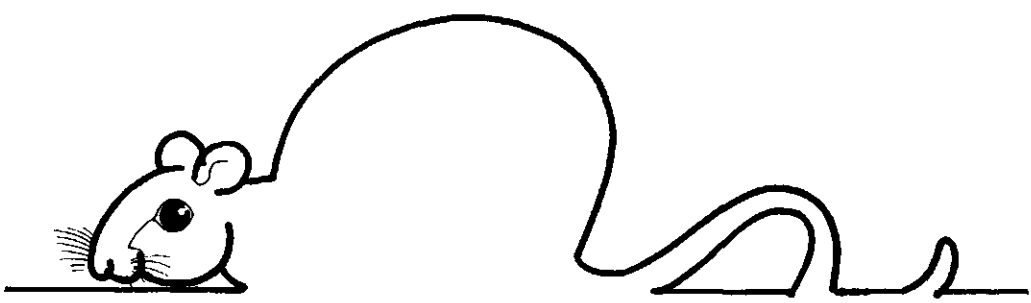
Synthetic leucine was added to the high protein meal in order to investigate the effect of specific leucine oversupply on metabolic leucine utilisation. During the first hour after the meal the percentage of labeled leucine subjected to decarboxylation was doubled. Since the very high protein meal showed a slower response, but provided a similar level of leucine, this indicates that free synthetic leucine is more rapidly absorbed and metabolised than protein-bound leucine.

It was suggested earlier that relative oversupply of amino acids increases the difference between CL and UL (Schreurs *et al.* 1992). Since CL and UL were significantly increased for seven hours after the meal, but CL-UL only for one hour, specific leucine oversupply appeared to induce both decarboxylation and total oxidation of leucine to the same extent. Thus the increase in the difference between CL and UL was more related to a general amino acid oversupply than a specific leucine oversupply (Schreurs *et al.* 1991). The small difference between CL and UL for the growing animals on a low protein diet is a typical illustration of the tight relation between amino acid availability and protein synthesis capacity. Earlier the same was shown for rats on a protein free diet (Schreurs *et al.* 1992).

Both the tracer and synthetic leucine were mixed through the meal as free leucine, which resulted in dilution of tracer. The specific activity of liver protein for the group with synthetic leucine was 0.71 times that without leucine (results not shown). This indicates that the specific activity, and therefore $^{14}CO_2$ output, for the group with synthetic leucine has been underestimated and should be increased with a factor (1/0.71=) 1.4.

Conclusion

The present study indicates that the level of chronic protein supply and growth do not or only marginally affect postprandial metabolic leucine utilisation. In contrast, the levels of dietary protein and leucine have dramatic adverse effects on postprandial metabolic leucine utilisation.



Chapter 4

EFFECT OF PREGNANCY ON POSTABSORPTIVE AND POSTPRANDIAL METABOLIC LEUCINE UTILISATION IN MATURE RATS

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The effect of pregnancy on postabsorptive and postprandial metabolic leucine utilisation was investigated in mature female rats (20 wks) before (non-pregnant, NP) and at day 18 of pregnancy (pregnant, P). Rats were conditioned on a high protein (HP) diet (210 g casein/kg feed). After overnight fasting rats were subjected to an 8 h $^{14}CO_2$ breath test during constant infusion of L-[1-^{14}C]-leucine ($^{14}CO_2$ output expressed as % infused dose). During the infusion a 5 g HP meal or low protein (LP) meal (75 g casein/kg feed; LP) was offered for 30 min. In the postabsorptive state $^{14}CO_2$ output was lower in P *v.* NP rats (12.5 (SE 0.7) % *v.* 15.9 (SE 1.1) %; $P<0.05$). Meal ingestion resulted in a fast increase in $^{14}CO_2$ output. After a HP meal $^{14}CO_2$ output (average 1.5 h to 5 h) was not different between P and NP rats (36.8 (SE 1.6) % *v.* 35.0 (SE 2.5) %). After a LP meal $^{14}CO_2$ output (average 1.5-5h) seemed lower in P *v.* NP rats. This contrast did not reach statistical significance (19.7 (SE 1.1) % *v.* 25.8 (SE 2.8) %). In conclusion, 1. pregnancy improved postabsorptive metabolic leucine utilisation, 2. seemed to improve postprandial metabolic leucine utilisation within limits of protein supply.

Amino acid metabolism: Leucine: Dietary protein: Pregnancy: Rat

INTRODUCTION

Naismith (1969) found that increased N-retention in the pregnant rat was primarily deposited in the maternal carcass (67%) in the first 2 weeks of pregnancy and in products of conception (84%) in the third (last) week of pregnancy. Naismith & Morgan (1976) than proposed to name these periods the anabolic and catabolic phases of pregnancy in the rat. Mayal-Afshar & Grimble (1983) found muscle protein to be increased in the anabolic phase, even on a protein deficient diet. Between day 17 and 20 of pregnancy Ling *et al.* (1987) found a reduction of muscle protein in well-fed rats, but Mayal-Afshar & Grimble (1983) only found a reduction of muscle protein in protein deficient rats. Also in the final week of pregnancy fetal and placental protein are increased substantially (Mayel-Afshar & Grimble, 1982, 1983; Ling *et al.* 1987, 1989). The normal increase in liver protein during pregnancy did not occur in protein deficient rats (Mayel-Afshar & Grimble, 1983). Although feed intake increases during pregnancy upto several days before delivery (Mayal-Afshar & Grimble, 1983; Moore & Brasel, 1984), the above suggests a complex repartitioning of protein between pools in addition to direct deposition of dietary protein. It has been suggested that amino acid oxidation might be decreased during pregnancy, like in protein deficiency (Naismith & Fears, 1972). Mayal-Afshar *et al.* (1981) found the percentage tyrosine oxidation to be decreased mainly in the catabolic phase, both in well-fed and protein-deficient rats.

In the present study we investigated metabolic leucine utilisation in the postabsorptive and postprandial state, using different protein meals.

MATERIALS AND METHODS

Animals and diets

Experiment 1. Mature female Wistar-WU rats (*n* 15; age, 20 wks; Centre for Small Laboratory Animals (CKP), Wageningen Agricultural University, Wageningen, The Netherlands) were housed in individual cages with bedding material (Woody-Clean type 8/15, Broekman Institute Ltd., Someren, The Netherlands). Room temperature was 22-23 °C. Light period was from 6.00 h to 20.00 h. Rats were offered feed between 9.00 h and 17.00 h for 4 weeks before the first experiment, and were conditioned on a high protein (HP) diet (210 g casein/kg feed) for 3 weeks before pregnancy. One week before the first breath test all rats were provided with a silicon heart catheter through the right jugular vein, for continuous infusion of tracer

(Steffens, 1969). The oestrus cycle was checked daily by vaginal lavages, in order to indicate the phase of oestrus cycle. Rats were first subjected to the $^{14}CO_2$ breath test at 2 days before pregnancy (day 2 or 3 of the di-oestrus phase; *n* 12; NP, non-pregnant) and second on day 18 of pregnancy (*n* 9; P, pregnant).

Experiment 2. In a pilot study female rats from the same batch (*n* 6) were conditioned on a low protein (LP) diet (75 g casein/kg feed). No breath tests were performed in this group (see RESULTS).

$^{14}CO_2$ Breath test

Experiment 1. Overnight fasted rats were twice subjected to an 8h infusion with L-[1-^{14}C]-leucine (2.0 GBq/mmol; Amersham, Den Bosch, The Netherlands). The isotope was diluted with deionized water and infused at a rate of 250 μl/h (4.6 kBq/h). Rats were either offered a HP meal (NP, *n* 6; P, *n* 5) or a LP meal (NP, *n* 6; P, *n* 4), if possible on both occasions. Three hours after start of infusion the 5 g test meal was offered for 30 min. The $^{14}CO_2$ output was measured continuously (15 min interval) and expressed as percentage of infused dose (**Chapter 1**).

Statistical analysis

Differences between NP and P rats were analysed for body weight, feed intake, weight gain and $^{14}CO_2$ output with Student's *t* test for paired or independent samples, where appropriate. Effects of pregnancy and protein content of the meal were analysed with ANOVA (SPSS Inc., 1988).

RESULTS

Experiment 1

Figure 1 shows that body weight was stable before pregnancy and progressively increased during pregnancy. Weight gain was most pronounced during the last week of pregnancy.

Table 1 shows significant increases in body weight, feed intake and weight gain at different stages during pregnancy. At day 18 of pregnancy feed intake was decreased but at the same time weight gain was tripled compared to preceding days.

Figure 2 shows that meal ingestion profoundly increased $^{14}CO_2$ output.

Table 2 shows that $^{14}CO_2$ output in the postabsorptive state (average -1 h to 0 h) was lower in P *v.* NP rats ($P<0.05$). In the postprandial state (average 1.5 h to 5 h) after a HP meal $^{14}CO_2$ output was not different between P and NP rats. After a LP meal

$^{14}CO_2$ output seemed lower in P *v.* NP rats, but this difference did not reach statistical significance.

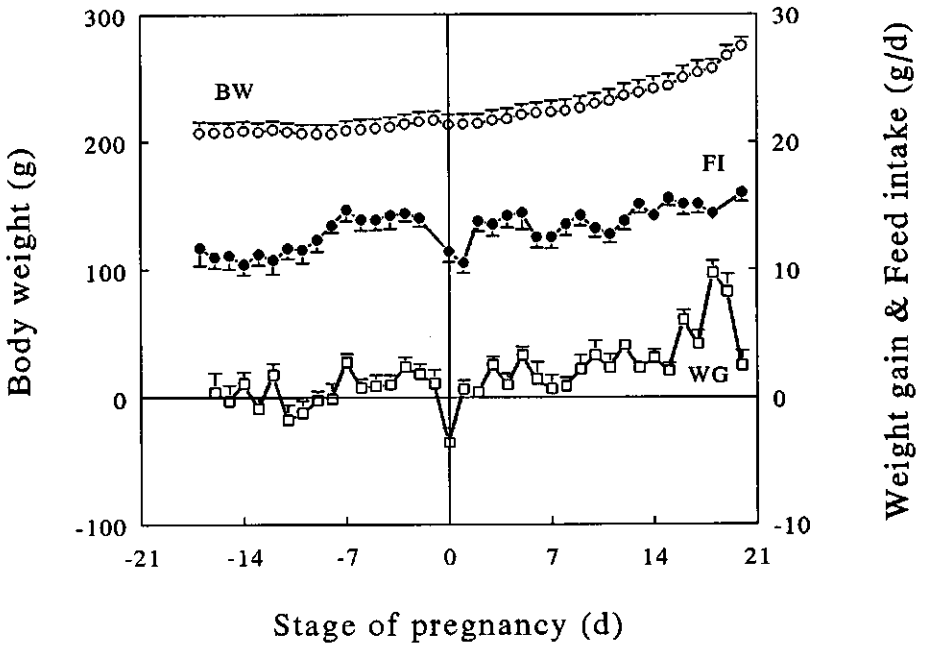


Figure 1. Development of body weight (open circles), feed intake (filled circles) and weight gain (open squares) before and during pregnancy (**Experiment 1**). Vertical bars indicate SE.

Table 1.
Body weight, feed intake and weight gain before and at different stages during pregnancy.

Period (d)	Body weight (g)	Feed intake (g/d)	Weight gain (g/d)
-14-0	210 ± 7[a1]	12.8 ± 0.4[a]	0.7 ± 0.1[a]
1-7	219 ± 8[b]	13.1 ± 0.6[ab]	1.4 ± 0.3[b]
8-14	233 ± 9[c]	13.8 ± 0.4[b]	2.6 ± 0.2[c]
15-17	250 ± 9[d]	15.2 ± 0.4[c]	3.1 ± 0.1[d]
18	264 ± 9[e]	14.4 ± 0.4[abc]	9.8 ± 0.8[e]

[1] Means in one column not charing a common character are significantly different ($P<0.05$).

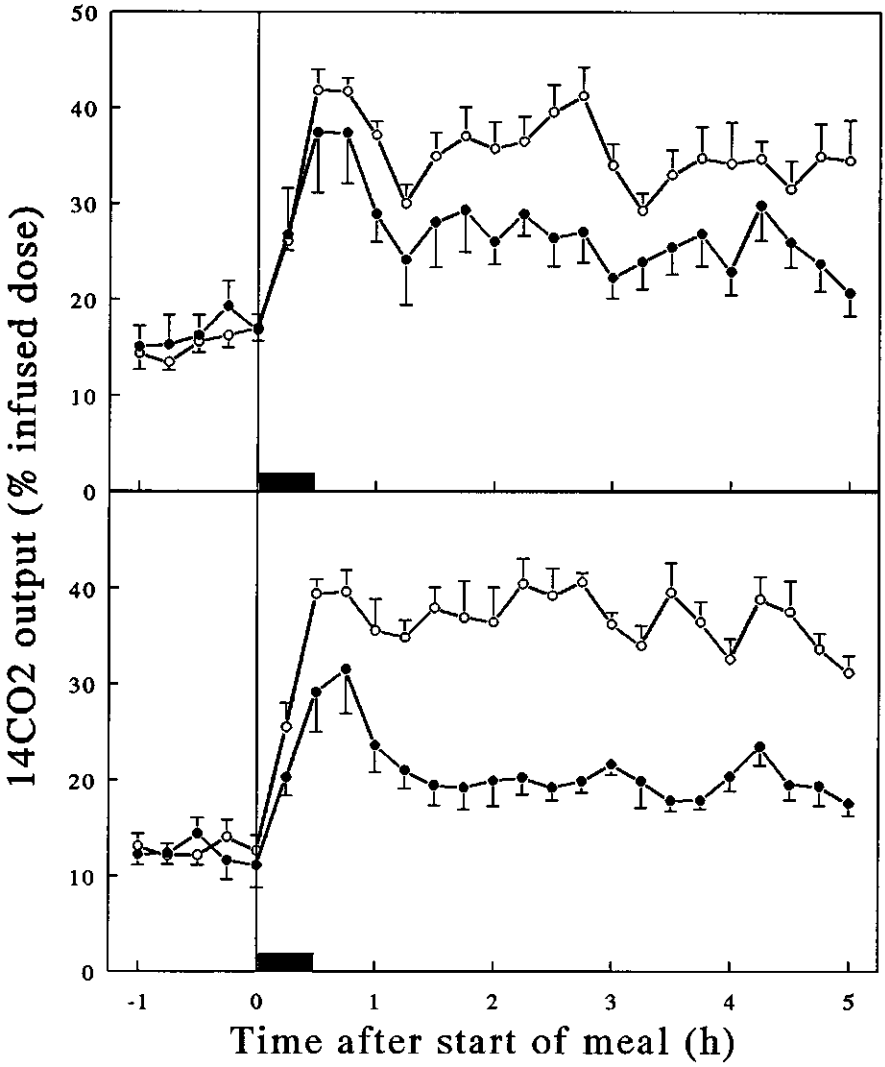


Figure 2. Meal-induced changes in $^{14}CO_2$ output before (upper panel) and during (lower panel) pregnancy (**Experiment 1**). Either a high protein meal (open circles) or a low protein meal (filled circles) was offered. Vertical bars indicate SE.

Experiment 2

Only 1 of 6 LP rats went through pregnancy and delivered. The litter size was 4 (all female), which is significantly less than for the HP diet (litter size **Experiment 1** was 8 to 12). This LP dame died at weaning age of the young, which were small and rather hareless. At 6 wks of age 2 of 4 young rats were changed to the HP diet. **Figure 3** shows that weight gain of these female HP rats was comparable to delayed weight gain of female Wistar-WU rats on a standard laboratory chow or even with male HP rats. Weight gain of the LP rats remained low, but later during development hair growth became normal on the LP diet. Body weight at 20 wks of age was 151 and 146 g for the LP rats *v.* 322 and 296 g for the HP rats, respectively.

Table 2.
Effect of pregnancy on metabolic leucine utilisation in the postabsorptive (PA) and postprandial (PP) condition.

Rat...	Non-Pregnant/Mature		Pregnant/Growing		ANOVA (P)[1]	
Meal...	High protein	Low protein	High protein	Low protein	P/G	Meal
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE		
Female rats						
PA	16.3 ± 1.1		12.9 ± 0.8#		0.001	NS
PP	35.1 ± 2.5	25.7 ± 2.6	36.7 ± 1.7	19.7 ± 1.0	NS	0.001
Male rats						
PA	14.6 ± 1.1		10.5 ± 0.6#		0.001	NS
PP	31.2 ± 1.7	19.7 ± 2.3	26.3 ± 1.4	15.9 ± 0.9	0.005	0.001

[1] Significant effect of pregnancy/growth (P/G) and meal according to analysis of variance.
Significantly different from mature rats (P<0.05).

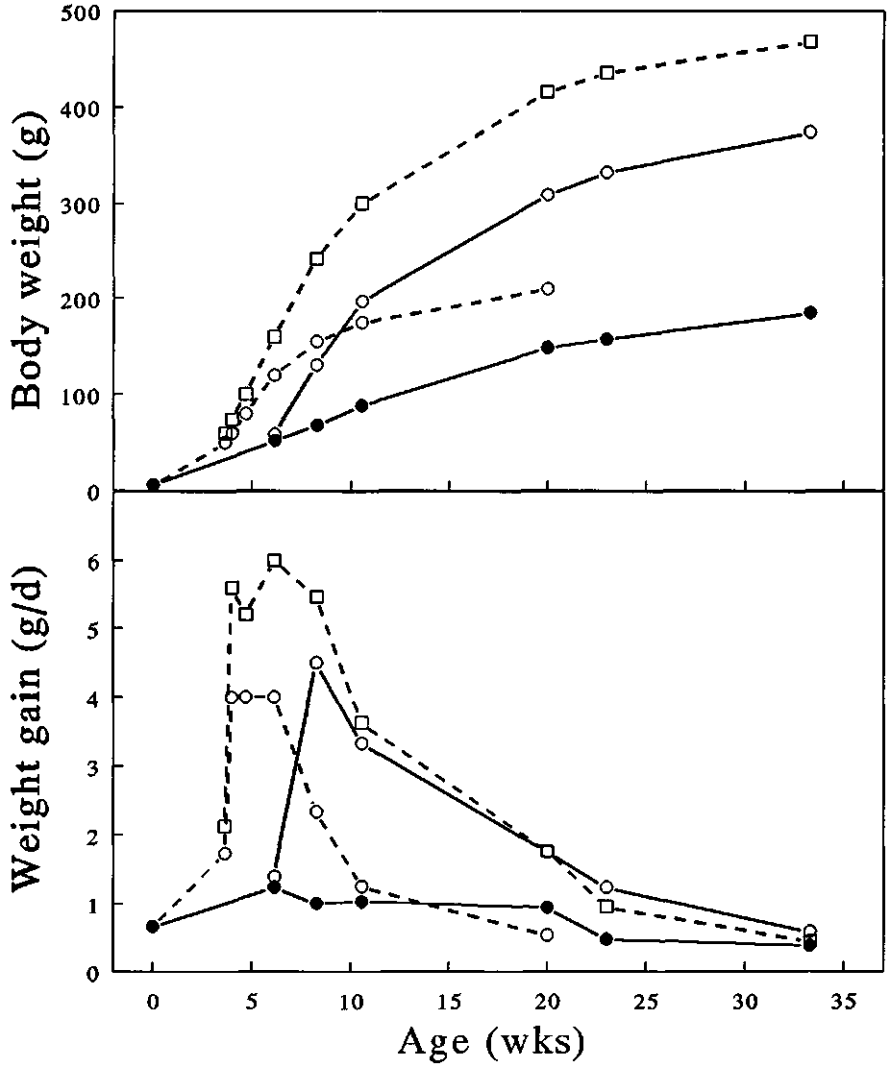


Figure 3. Development of body weight (upper panel) and weight gain (lower panel) for high protein female rats (open circles) and low protein female rats (filled circles) (**Experiment 2**). For comparison female rats on standard laboratory chow (open circles, dashed line) and high protein male rats (open squares, dashed line) are shown.

DISCUSSION

Introduction

Although pregnancy is an interesting physiological phenomenon in itself, the primary objective of the present study was to check whether growth during pregnancy has comparable effects on metabolic leucine utilisation as growth during development. In an earlier study with male rats (**Chapter 1**; summarized in **Table 2**), growth significantly improved metabolic leucine utilisation in the early postprandial phase as well as in the postabsorptive state.

During pregnancy the mature female rat seems to gain muscle protein in the first two weeks (Poo *et al.* 1940; Naismith, 1969; Mayal-Afshar & Grimble, 1983), and placental and fetal protein in the last week of pregnancy (Mayel-Afshar & Grimble, 1982, 1983; Ling *et al.* 1987, 1989). In the last week of pregnancy the placental and fetal tissues seem to gain protein at the expense of muscle tissue protein (Naismith, 1969; Ling *et al.* 1987), although this might only be necessary on protein deficient diets (Mayal-Afshar & Grimble, 1983).

Recently Millward & Rivers (1988) proposed that diurnal protein cycling in the mature animal would account for postprandial protein gain and postabsorptive protein loss. Transient protein gain after feeding is likely to occur in gut and liver, which can subsequently supply amino acids to muscle tissue (Simon, 1989). Maternal muscle protein gain after feeding with subsequent amino acid release in the postabsorptive state has been suggested to be essential to fetal development (Ling *et al.* 1987, 1989). When amino acid release in the postabsorptive state was reduced by continuous feeding, fetal growth was retarded (Ling *et al.* 1989).

Effect of pregnancy postprandially

It remains to be shown that normal transient protein gain after feeding fully accounts for the fetal amino acid needs in the postabsorptive state. When normal transient protein gain on a high protein diet is high enough for this purpose, there is no need to improve metabolic leucine utilisation after a high protein meal. The present results support this hypothesis, since metabolic leucine utilisation after a high protein meal was not improved for pregnant rats. However, after a low protein meal metabolic leucine utilisation seemed to be improved in the pregnant rat. This indicates that when less dietary protein is available, the efficiency of utilisation is increased. In this way the pregnant rat tries to gain as much protein as after a high protein meal. This is essentially the same as we previously observed (**Chapter 1**) with growing rats in comparison to mature rats (**Table 2**). Although in growing rats metabolic leucine

utilisation was even improved after the high protein meal.

Effect of pregnancy postabsorptively

In the postabsorptive state the transient protein gain after feeding is mobilised by protein breakdown and amino acids are released into the metabolic pool. Pregnancy reduced the percentage leucine oxidation in the postabsorptive state (**Table 2**), which indicates that metabolic leucine reutilisation is improved. This improvement was also found for growing rats in comparison to mature rats (**Table 2; Chapter 1**).

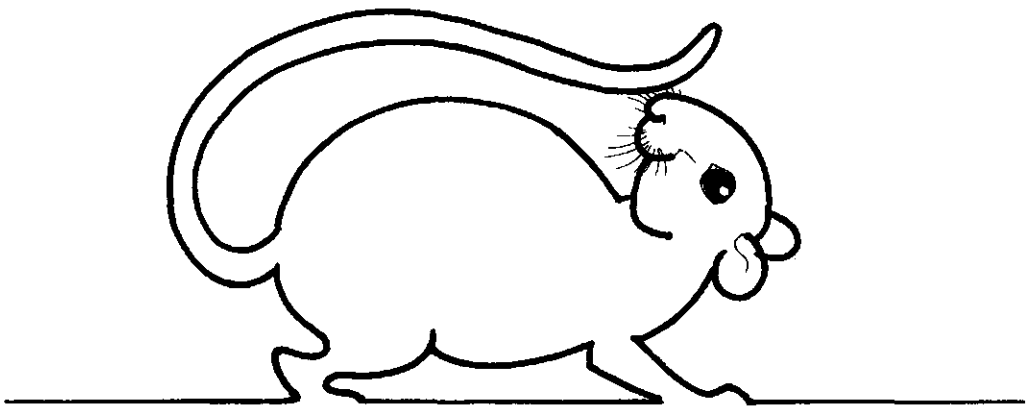
Effect of growth

Thus the growth effect of pregnancy does confirm the observed effect of developmental growth, namely improved postabsorptive and postprandial metabolic leucine utilisation. This means that a higher percentage of leucine flux is directed towards protein synthesis, at the expense of oxidative processes. Almost immediately after refeeding with a low protein diet, the percentage leucine oxidation seemed to be governed by acute protein supply.

Limits to adaptation

That there are limitations to this mechanism of adaptation is clearly shown by **Experiment 2.** It became clear that the low protein diet did not meet normal pregnancy requirements, although much lower protein diets have been shown to result in normal pregnancy (Nelson & Evans, 1953). One of the 6 rats delivered and fed 4 youngs untill weaning age, but at dramatic costs. The effect of early protein deprivation (during pregnancy and upto 3 wks after weaning) on weight gain seemed to be highly reversible (compensatory growth) with a high protein diet (**Figure 3**). Weight gain of these two high protein female rats became comparable to weight gain of female Wistar-WU rats, although with delayed onset. Because data for the high protein diet were not available for the female rat, we have added information for the male rat. Weight gain of the two high protein female rats was comparable with the high protein male rats. The two low protein female rats showed a minimal growth rate.

This does not just show the magnificent adaptation during pregnancy, but it increases the likelyhood of transient muscle protein gain and loss in mature animals. When this can be assessed adequately it could well be the mechanism that fails with muscle wasting in several clinical conditions, including the severely ill patient.



Chapter 5

EFFECTS OF MEAL FREQUENCY AND TRACER INFUSION TIME ON METABOLIC LEUCINE UTILISATION IN MATURE RATS

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Effects of meal frequency and tracer recycling on metabolic leucine utilisation measurements. Rats were conditioned on a liquid feed (40 ml/day) for 6 days. In a cross-over design, either 2 meals of 20 ml (low meal frequency, LMF; 0 and 5 h) or 5 meals of 8 ml (high meal frequency, HMF; 0, 2, 4, 6 and 8 h) were offered within a 10 h feeding period. At day 7, rats were subjected to a 20-22 h $^{14}CO_2$ breath test during a primed constant infusion of L-[1-^{14}C]-leucine. For both meal frequencies tracer infusion started either 2 h (group A) or 10 h (group B) before feeding. Before feeding $^{14}CO_2$ output was higher for HMF *v.* LMF ($P<0.05$). Ingestion of a meal profoundly increased $^{14}CO_2$ output. During feeding $^{14}CO_2$ output was lower for HMF *v.* LMF at hour 2 of feeding ($P<0.1$), but higher at hour 5 and 9 ($P<0.001$). After feeding (hour 10-18) $^{14}CO_2$ output was higher for HMF *v.* LMF ($P<0.005$). After feeding $^{14}CO_2$ output was profoundly increased above the level before feeding, indicating that tracer recycling contributed substantially to $^{14}CO_2$ output. When tracer infusion time before feeding was increased, $^{14}CO_2$ output profoundly increased before feeding ($P<0.001$), but not during feeding. After feeding $^{14}CO_2$ output was only increased for LMF ($P<0.001$). In conclusion, metabolic leucine utilisation changed acutely with meal feeding, but differently for meal frequencies. It is suggested that larger meals result in more profound changes in protein breakdown. Profound tracer recycling was found with long tracer infusion times in the fasted state, but not during feeding.

Amino acid metabolism: Leucine: Meal frequency: Recycling: Rat

INTRODUCTION

In rats forced feeding two meals versus ad libitum feeding has been shown to increase 24h urinary urea N output by 38% (Cohn *et al.* 1963). In humans 24 h N excretion was not significantly different between 2 and 7 meals/day (Verboeket-van de Venne & Westerterp, 1991), 1 and 5 meals/day (Wolfram *et al.* 1987), and continuous and intermittent nasogastric feeding (Heymsfield *et al.* 1987). In growing pigs live weight gain, feed conversion ratio and lean in ham were not different between 1 and 6 meals per day of a lysine deficient diet. However, when the feed was supplemented with free lysine the increase in live weight gain was lower with 1 versus 6 meals per day (Batterham, 1974; Batterham & O'Neill, 1978).

Several investigations have shown diurnal variation in amino acid oxidation or nitrogen excretion. In adult humans, leucine oxidation was higher with feeding compared to fasting (Garlick *et al.* 1980; Motil *et al.* 1981; Clugston & Garlick, 1982; Rennie *et al.* 1982; Hoffer *et al.* 1985; Melville *et al.* 1989; Millward *et al.* 1991) and increased with dietary protein intake (Motil *et al.* 1981; Price *et al.* 1990). In these studies repeated small meals were used in order to create a steady absorptive state. Larger meals may have a different effect on metabolic amino acid utilisation, because of metabolic limitations to protein deposition. In the mature animal 24 h N-balance will not improve with a higher frequency of feeding, since it is in N-equilibrium. However, diurnal protein cycling might be different.

The study of diurnal variations in amino acid utilisation may need long tracer infusion times. A 24 h tracer infusion has been shown to underestimate leucine flux by 25% (Schwenk *et al.* 1985). Due to design they did not show when significant recycling occurred. A 24 h tracer infusion has been shown to underestimate leucine flux by 15% in the fasted state, but not in the fed state (Melville *et al.* 1989).

In the present study both the effect of meal frequency and tracer infusion time on metabolic leucine utilisation were investigated.

MATERIALS AND METHODS

Animals and diets

Mature male Wistar-WU rats (*n* 15; 351 g; 35 wks; Center for Small Laboratory Animals, Wageningen Agricultural University, Wageningen, The Netherlands) were housed in individual cages with a wired bottom. Room temperature was 22-23°C. Light period was from 6.00 h - 20.00 h.

Rats were fed 40 ml liquid feed per day (casein hydrolysate, 1.6 g; energy, 168 kJ; Nutrison Standard, Nutricia Lab., Zoetermeer, The Netherlands). This volume was ingested by rats when they were fed two times per day for 30 min, with no feed restriction. Within a 10h feeding period 2 meals of 20 ml (low meal frequency, LMF) were offered at 10.00h (0 h) and 15.00 h (5 h) or 5 meals of 8 ml (high meal frequency, HMF) were offered at 10.00 h (0 h), 12.00 h (2 h), 14.00 h (4 h), 16.00 h (6 h) and 18.00 h (8 h) for 6 days. Rats were subjected to both meal frequencies according to a cross-over design.

$^{14}CO_2$ Breath test

At day 7 of each dietary period rats were subjected to a 20-22 h primed constant infusion of L-[1-^{14}C]-leucine (prime, 1.4 kBq; 18.5 kBq/ml; 150 ul/h; 2.0 GBq/mmol; Amersham, Den Bosch, The Netherlands). Infusion started either 2 h (group A) or 10 h (group B) before feeding. Expired $^{14}CO_2$ was trapped in KOH (250 ml; 0.3 mol/l). Samples were taken every 15 min and were analysed by liquid scintillation counting. Expired $^{14}CO_2$ was expressed as percentage of infused dose (**Chapter 1**). Results are presented as hourly average of 4 samples.

Statistical analysis

Data were subjected to analysis of variance and Student's *t* test for independent or paired samples where appropriate (SPSS Inc., 1988).

RESULTS

Body weight and feed intake

Feed intake prior to the experiment was about 216 kJ/d (12 g/d; 18 kJ/g), corresponding to 50 ml Nutrison. Rats, allowed to eat twice per day for 30 min an unrestricted volume of intake, consumed 19.7 (SE 1.0) ml with the first meal and significantly more with the second meal (22.6 (SE 0.4) ml; $P<0.05$). During an 8 d period rats did not increase intake with the first meal. Therefore the daily ration of 40 ml Nutrison was used in this experiment. Since body weight slightly decreased during the experiment this intake can be considered as slightly restricted in protein and/or energy (**Figure 1**). Only weight loss of rats on the LMF regime in the second week was significant ($P<0.05$).

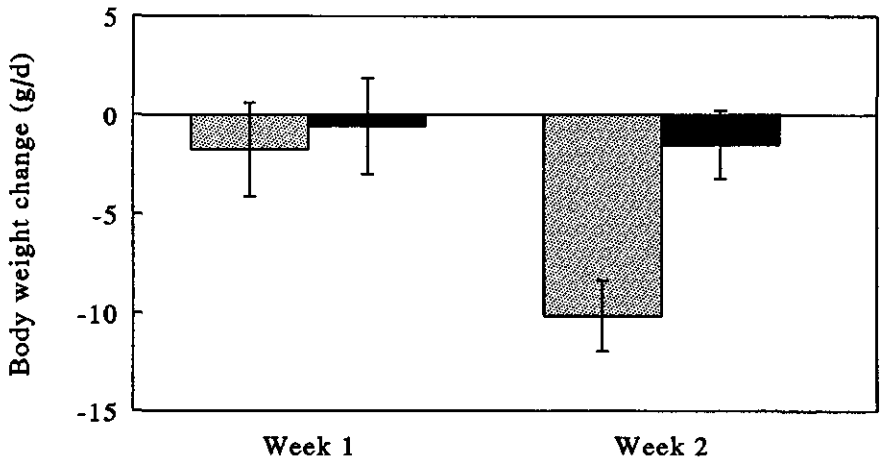


Figure 1. Body weight changes during week 1 and 2 on the low meal frequency feeding regime (grey bars) and high meal frequency feeding regime (black bars). Vertical bars indicate SE.

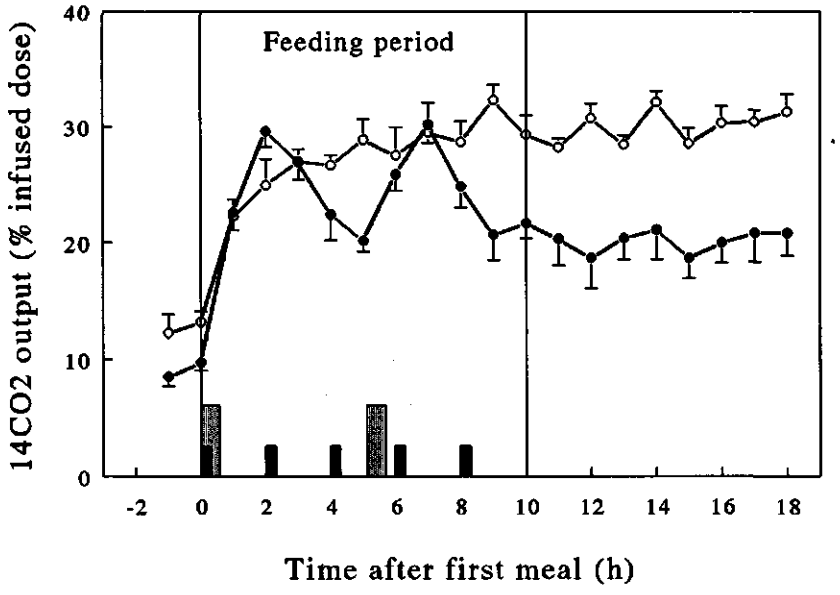


Figure 2. Changes in $^{14}CO_2$ output for the low meal frequency (LMF) feeding regime (filled circles) and the high meal frequency (HMF) feeding regime (open circles). Vertical bars indicate SE.

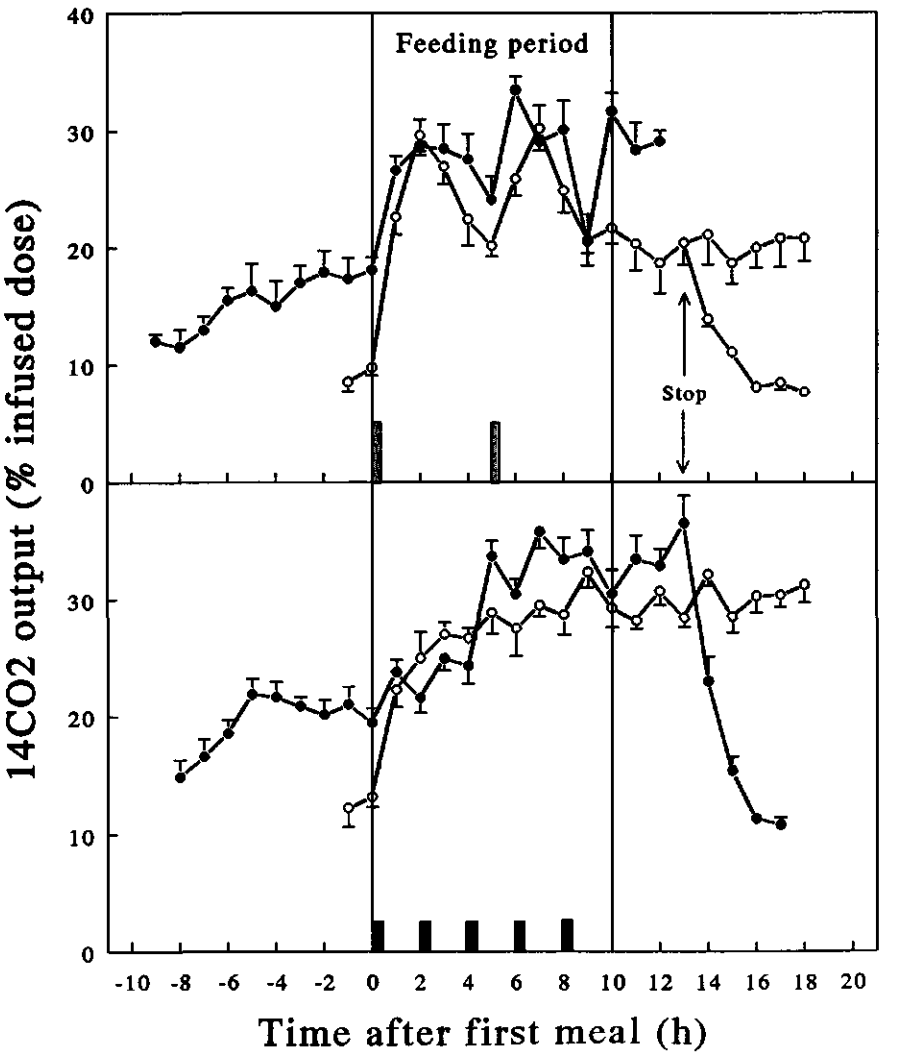


Figure 3. Changes in $^{14}CO_2$ output for the low meal frequency (LMF) feeding regime (upper panel), and the high meal frequency (HMF) feeding regime (lower panel). Group A, tracer infusion started 2 h before the feeding period (open circles); group B, tracer infusion started 10 h before the feeding period (filled circles). Vertical bars indicate SE.

$^{14}CO_2$ Breath test

Figure 2 shows that before feeding $^{14}CO_2$ output was higher for HMF *v.* LMF ($P<0.05$) (1.35 times). Ingestion of a meal profoundly increased $^{14}CO_2$ output. During feeding $^{14}CO_2$ output was lower for HMF *v.* LMF at hour 2 of feeding ($P<0.1$), but higher at hour 5 and 9 ($P<0.001$). After the feeding period (hour 10-18) $^{14}CO_2$ output was higher for HMF *v.* LMF ($P<0.005$) (1.47 times). After feeding $^{14}CO_2$ output was profoundly increased above the level before feeding (HMF, 2.13 times; LMF, 2.38 times).

Figure 3 shows that increased tracer infusion time before feeding (group B), profoundly increased $^{14}CO_2$ output before feeding ($P<0.001$), but not during feeding. After feeding $^{14}CO_2$ output was only increased for LMF ($P<0.001$) (**Figure 3a**). It also shows that when the infusion was stopped at hour 13 $^{14}CO_2$ output declined to 8.1% for LMF ($n=3$) and 11.4% for HMF ($n=8$) (1.41 times).

DISCUSSION

Effect of meal frequency

The percentage of labeled leucine subjected to decarboxylation has been shown to increase after ingestion of a meal (**Chapter 1**). In that investigation pelleted feed was used with different protein content. The increase in the percentage leucine decarboxylation was much higher after a high protein meal compared to a low protein meal. Since it was suggested that the metabolic response was due to the amount of ingested protein, changes in meal size should result in similar responses. Without a change in daily intake the change in meal size is associated with a change in meal frequency.

Meal frequency has been shown to affect protein metabolism (Cohn, 1963), though not significant in most cases (Heymsfield *et al.* 1987; Wolfram *et al.* 1987; Verboeket-van der Venne & Westerterp, 1991). Especially in the mature animal it would be difficult to find any change in 24 h N-balance. Since the mature animal is in N-equilibrium, daily efficiency of dietary amino acid utilisation is essentially zero. However, postprandial amino acid utilisation, and especially postprandial protein deposition, may be improved and thus the amplitude of diurnal protein cycling would increase (Millward & Rivers, 1988).

Presently we have only investigated the acute effect of large and small meals, with corresponding meal frequency, on the percentage leucine decarboxylation. Previously we showed that metabolic leucine utilisation is largely determined by acute protein

supply (**Chapter 1**).

After an overnight fast the percentage leucine decarboxylation was lower for low meal frequency feeding (large meals) in the mature rats, which indicates a higher efficiency of utilisation. It suggests that less protein is deposited during the previous feeding period, resulting in a smaller amplitude in diurnal protein cycling.

The percentage leucine decarboxylation remained lower for the low meal frequency feeding (large meals), both during and after feeding. After a single small meal this percentage decreased again (data not shown), but with repeated small meals this fall is prohibited. After a large meal this fall was still found. A more continuous absorptive state can be expected to result in smaller metabolic changes. The fact that the efficiency was higher for the low meal frequency feeding does not necessarily mean, that it would be more efficient on a daily basis. In fact, when it is assumed that this metabolic efficiency is relevant to dietary protein (Hoerr *et al.* 1991), the dietary leucine flux is higher after a large meal and therefore the absolute value of leucine decarboxylation is higher. Furthermore the free leucine pool size might also be larger, which results in a lower specific activity of the precursor pool and thus a lower $^{14}CO_2$ output. But even taking these aspects into account, it was still expected that the efficiency would be lower with the larger meals. What could be seen however were acute changes in efficiency, which were quite substantial with large meals but hardly detectable with small meals.

Repeated small meals have been shown in humans to decrease protein breakdown (McNurlan & Garlick, 1989). This decrease was even larger with a higher protein intake (Motil *et al.* 1981). Especially liver protein breakdown seemed to be responsive to feed intake since it increased in the early postabsorptive state, and even more after 24 h fasting (Mortimore & Pösö, 1987). Both gut and liver protein have been suggested as short term protein storage (Simon, 1989). The difference in metabolic response to a large or small meal may well be a difference in the decrease of protein breakdown. Especially since mature animals are not able to increase protein synthesis (Baillie *et al.* 1988; Garlick *et al.* 1990), contrary to growing animals (Garlick *et al.* 1983; Garlick & Grant, 1988). Since a larger percentage of leucine was used for protein synthesis after a large meal compared to a small meal, protein synthesis does not seem to be limiting. It suggests that protein breakdown is more suppressed by large meals than small meals.

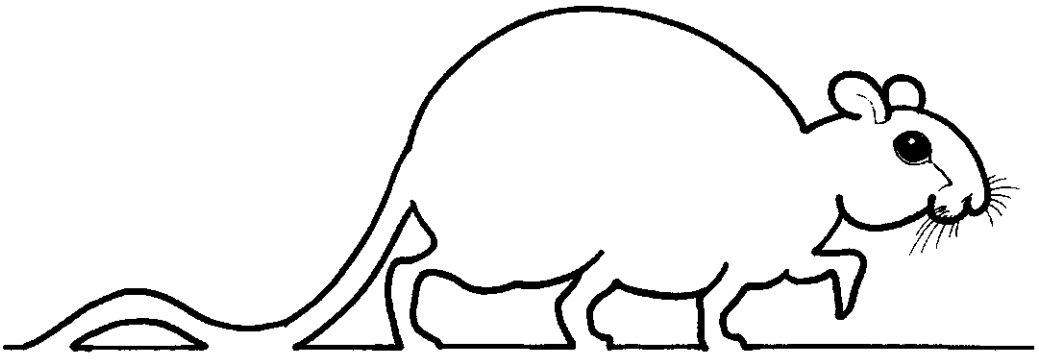
Effect of tracer infusion time

In group A (2 h pre-feeding infusion) $^{14}CO_2$ output was much higher after feeding than before feeding, although there should be no physiological difference. Evidently

recycling accounted for a large part of $^{14}CO_2$ output after several hours of infusion.
A 24 h tracer infusion has been shown to underestimate leucine flux by 25%, due to recycling of label (Schwenk *et al.* 1985). They could not show when significant recycling occurred, but suggested as soon as 4 h of infusion. It was shown in humans that significant recycling occurred after 5 h infusion (Abumrad *et al.* 1984).
When tracer infusion was started 10 h before feeding (group B) instead of 2 h (group A) $^{14}CO_2$ output during the first hours of infusion was slightly higher. This indicates that the percentage leucine decarboxylation slightly decreased during the overnight fast. More important however was the profound difference in $^{14}CO_2$ output between group A and B just before feeding, for both meal frequencies. This indicates substantial recycling with longer tracer infusions in the fasted state.
A 24 h tracer infusion has been shown to underestimate leucine flux by 15% in the fasted state, but not in the fed state (Melville *et al.* 1989). When tracer infusion was stopped after 12 h feeding, plasma leucine and ketoisocaproate enrichment were still 8-10% of feeding plateau after 10-12 h fasting. Thus recycling can play an important role in leucine oxidation measurements, but this is almost abolished during feeding. This must be largely due to a decrease in protein breakdown during feeding (Garlick *et al.* 1990). That groups A and B did not show a different $^{14}CO_2$ output during feeding indicates that recycling is not important during feeding and this suggests that protein breakdown is decreased.
Towards the end of the feeding period, hour 4 after the second large meal, $^{14}CO_2$ output was not different between group A and B. However, the next hour (hour 10) $^{14}CO_2$ output increased drastically to a much higher level for LMF. This is an indication that protein breakdown increased again in the early postabsorptive state, as has also been described for liver protein breakdown (Mortimore & Pösö, 1987).
The longer tracer infusion times resulted in increased recycling after feeding, but this was only shown for the LMF. It is suggested that a longer tracer infusion time in the fasted state favours the labelling of protein pools with a low turnover rate (like muscle). Since the effect of the longer tracer infusion time during the fasted state was not shown for high meal frequency feeding, this also suggests that more label is initially incorporated into e.g. muscle. Proteins with a low turnover rate will only marginally contribute to recycling.
When tracer infusion was stopped several hours after the feeding period, recycling accounted 8.1% for the low meal frequency feeding and 11.4% for the high meal frequency feeding. Although the difference is small, the HMF/LMF ratio is similar to that in group A both before and after feeding.

Conclusion

Metabolic leucine utilisation changed acutely with meal feeding, but differently for meal frequencies. It is suggested that larger meals result in more profound changes in protein breakdown. It is also suggested that profound tracer recycling occured with long tracer infusion times in the fasted state.



Chapter 6

EFFECTS OF TRAINING AND CHRONIC PROTEIN RESTRICTION ON METABOLIC LEUCINE UTILISATION AFTER EXERCISE AND FEEDING

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Effects of training and chronic protein restriction on metabolic leucine utilisation after exercise and feeding were investigated. Growing rats (10 wks) were either conditioned on a high protein (HP) diet (210 g casein/kg feed) or a low protein (LP) diet (75 g casein/kg feed). Rats were either sedentary (control) or trained on a treadmill (20 m/min, 60 min/d, 5 d/wk, 5 wks). An 8 h $^{14}CO_2$ breath test was performed during a primed constant infusion of L-[1-^{14}C]-leucine (CL) or L-[U-^{14}C]-leucine (UL); $^{14}CO_2$ output was expressed as percentage of infused dose. During the infusion rats were challenged with a 1 h swim (water temperature, 35°C; tail load, 3 g/100 g rat) or a 5 g HP meal (30 min). Before the challenge $^{14}CO_2$ output was lower for the LP *v.* HP diet ($P<0.001$), both for CL and UL. After exercise the diet effect remained significant ($P<0.001$), but disappeared after the HP meal. During the first hour after exercise both $^{14}CO_2$ output and plasma leucine concentration increased above the pre-exercise level ($P<0.05$). Training tended to decrease $^{14}CO_2$ output after exercise for the HP group and increase it for the LP group. Training also decreased $^{14}CO_2$ output at hour 6 after feeding for the HP group. In conclusion, 1. chronic protein restriction improved metabolic leucine utilisation in the postabsorptive state, both before and after exercise, 2. training improved metabolic leucine utilisation in the high protein group both in the post-exercise and postprandial state, and 3. it is suggested that adaptation to training is especially relevant to the low protein diet.

Amino acid metabolism: Leucine: Dietary protein: Feeding: Training: Exercise: Rat

INTRODUCTION

About 150 years ago, Justus von Liebig firmly believed that body protein was the sole fuel for muscular work (Munro, 1964). Although it is now clear that mainly carbohydrates and fat provide the energy for muscular work, the importance of protein breakdown and amino acid oxidation remains unclear (Cathcart, 1925; Paul, 1989). In humans leucine flux (protein breakdown) was found unchanged during (Rennie *et al.* 1981; Hagg *et al.* 1982; Wolfe *et al.* 1982; Knapik *et al.* 1991) and after exercise (Devlin *et al.* 1990). Leucine oxidation however was found increased during exercise in rats (White & Brooks, 1981; Lemon *et al.* 1982; Lemon *et al.* 1985), and in humans (Rennie *et al.* 1981; Hagg *et al.* 1982; Wolfe *et al.* 1982; Knapik *et al.* 1991). However, other studies in humans have reported no change in leucine oxidation during and after exercise (Tarnopolsky *et al.* 1991) and a decrease in leucine oxidation after exercise (Devlin *et al.* 1990).

In general protein breakdown did not seem to change with exercise, while leucine oxidation was increased. This can be explained by a decrease in protein synthesis during exercise (Viru, 1987), which reduces the efficiency of reutilisation. The increase in leucine oxidation during exercise is not reflected by an increase in urea production (Wolfe *et al.*, 1984). Therefore reutilisation of amino acids is decreased during exercise, without a change in nitrogen reutilisation. The effect of exercise on nitrogen excretion may depend on the time after feeding (Cuthbertson *et al.* 1937).

Gontzea *et al.* (1975) showed a negative nitrogen balance at onset of training, but Todd *et al.* (1984) showed that training improved the nitrogen balance in humans. In rats it was shown that training increased both leucine flux (protein breakdown) and leucine oxidation, both at rest and during exercise (Dohm *et al.* 1977; Henderson *et al.* 1985). While the percentage leucine oxidation was not changed at rest and lower during exercise (Henderson *et al.* 1985).

The impact of these observations may depend on the nutritional condition and diurnal changes in metabolic leucine utilisation. Normal diurnal protein cycling involves postprandial protein gain and postabsorptive protein loss (Millward & Rivers, 1988). When exercise increases leucine oxidation in the postabsorptive state, this indicates an increased protein loss. Adaptation to training may involve a decreased postabsorptive protein loss or an increased postprandial protein gain.

In the present study the effects of chronic protein restriction and training on exercise- and meal-induced changes in metabolic leucine utilisation were investigated.

MATERIALS AND METHODS

Animals and diets

Male Wistar-WU rats (Harlan-Cpb, Zeist, The Netherlands) were housed in individual cages with bedding material (Woody-Clean type 8/15, Broekman Institute Ltd., Someren, The Netherlands). Room temperature was 22-23°C. Rats were conditioned on a high protein (HP) diet (210 g casein/kg feed) or a low protein (LP) diet (75 g casein/kg feed), starting at age 4 wks. Rats were trained from age 5 to 10 wks. Training involved treadmill (COTM, Bialystok, Poland) running at 20 m/min, 60 min/d, 5 d/wk (extra training in weekend before breath tests). Rats were provided with a silicone canula in the subclavian vein at the age of 9 wks for continuous infusion of tracer and sampling of blood (Steffens, 1969).

$^{14}CO_2$ Breath test

Rats were subjected to three 8 h primed constant infusions of labeled leucine. Tracers, L-[1-^{14}C]-leucine (carboxyl, CL) and L-[U-^{14}C]-leucine (universal, UL) (Amersham, Den Bosch, The Netherlands), were diluted with deionized water and infused at a rate of 250 ul/h. First and second, rats were infused with CL (prime, 4.6 kBq; 9.25 kBq/h; 2.0 GBq/mmol), and third with UL (prime, 18.5 kBq; 37 kBq/h; 2.0 GBq/mmol) with 2-3 days in between. Two h after start of the infusion rats were challenged with a meal or a swim. The expired $^{14}CO_2$ was trapped in a KOH solution (100-150 ml; 2 mol/l), which was refreshed every 2 h. Samples of 2 ml were taken every 15 min and 1 ml was mixed with 10 ml scintillation cocktail (Ultima Gold, Packard Inc., Groningen, The Netherlands). Expired $^{14}CO_2$ was expressed as percentage of infused dose.

Rats were fasted for 30 h before they were offered a 5 g HP meal for 30 min. The meal was provided after 2 h of tracer infusion.

Rats were ad libitum fed untill 6 h before they were subjected to 1 h swimming exercise. The temperature of the water was 35°C and a load of 3 g/100 g rat was fixed to the tail. After swimming exercise rats were wrapped in a warm towel and firmly dried with tissues. The tail weight was removed and rats were back in their dry cages within 2 min.

It was tried to reduce the acute stress response to first time swimming (Scheurink *et al.* 1989) by acquainting the rats with the swimming procedure 1 day before the experiment, for 10-15 min.

Those first 10 min were also the most active period of swimming. Furthermore the rats remained to show cleaning activity untill 30-45 min after swimming exercise. In

order to account for both activities some rats (LP group; $n=5$) were subjected to 10 min swimming exercise. A control group (no treatment; LP group; $n=3$) was used to account for any trend during the infusion time not related to swimming exercise. These experiments were only performed with CL.

On a different occasion blood samples were taken before, at 45 min of swimming, 45 and 105 min after swimming. Blood samples were spinned down and plasma was stored at -20°C for later amino acid analysis (Biotronik LC6000E, Biotronik, Frankfurt, Germany).

Statistical analysis

Data were subjected to ANOVA and group means were compared with Student's *t* test for independent or paired samples where appropriate (SPSS Inc., 1988).

RESULTS

Body weight and feed intake

Table 1 shows that body weight is lower for the LP *v.* HP group ($P<0.001$). Body weight and feed intake was lower for trained *v.* control rats of the HP group ($P<0.005$). Feed intake per kg body weight however was not different between trained and control rats.

Table 1.
Body weight and feed intake.

Diet...	High protein		Low protein	
Rats...	Control	Trained	Control	Trained
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Body weight (g)	327 ± 5	298 ± 7#	144 ± 5	152 ± 5
Feed intake (g/d)	22.2 ± 0.6	18.8 ± 0.6#	12.4 ± 0.6	12.5 ± 0.5
Feed intake (g/kgBW.d)	0.75	0.71	0.96	0.98

Significantly different from control group.

$^{14}CO_2$ Breath test

Table 2 shows that before the swim (hour 0) $^{14}CO_2$ output was lower for the LP *v.* HP group ($P<0.001$). This diet effect remained after 1 h swimming (hour 2, 4 and 6; $P<0.005$), both for CL and UL. In the first hour after exercise (hour 2) $^{14}CO_2$ output increased above the pre-exercise level ($P<0.05$). The plasma leucine concentration also increased significantly at 45 min after exercise (HP group: 0.141 (SE 0.020) μmol/ml *v.* 0.121 (SE 0.008) μmol/ml; LP group: 0.103 (SE 0.006) μmol/ml *v.* 0.086 (SE 0.007) μmol/ml; $P<0.05$).

Figure 1 shows that rats subjected to 10 min swimming showed a largely similar increase in $^{14}CO_2$ output in hour 1 (average of 30 and 45 min sample) after exercise. The $^{14}CO_2$ output level remained increased above the basal level to hour 6 after onset of swimming ($P<0.05$).

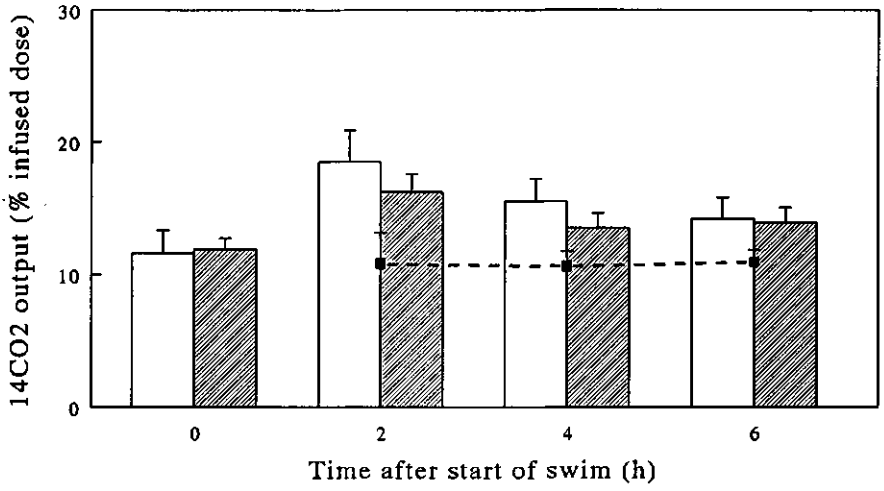

Figure 1. Changes in $^{14}CO_2$ output (% infused dose) for 60 min swim (tight dashed bars), 10 min swim (sparse dashed bars) and control rats (no treatment). Vertical bars indicate SE.

Table 2 shows that $^{14}CO_2$ output after exercise is different between the HP and LP group for control rats, but not for trained rats. Training tended to decrease $^{14}CO_2$ output for the HP group and increase $^{14}CO_2$ output for the LP group.

Table 3 shows that before the meal (0 h) $^{14}CO_2$ output was lower for the LP *v.* HP group ($P<0.001$), both for CL and UL. After ingestion of a HP meal (hour 2, 4 and 6) $^{14}CO_2$ output increased very fast and the effect of chronic protein restriction

disappeared. For the HP group $^{14}CO_2$ output decreased ($P<0.05$) at hour 6 *v.* hour 2 and 4 after the HP meal. This was due to a significant decrease in the trained group ($P<0.05$), but not in the control group.

Table 2.
Effects of diet and training on exercise-induced changes in $^{14}CO_2$ output.

Diet...	High protein		Low protein		ANOVA (P)[1]	
Rats...	Control	Trained	Control	Trained	Diet	Rats
Time	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE		
			L-[1-^{14}C]-leucine			
	(n=5)	(n=6)	(n=5)	(n=7)		
0	17.5 ± 1.7^{a2}	17.0 ± 0.8^{a}	10.7 ± 1.5^{a}	11.6 ± 1.7^{a}	0.001	NS
2	27.1 ± 1.3^{b}	22.1 ± 1.6^{b}	16.9 ± 1.9^{b}	18.5 ± 2.4^{b3}	0.004	NS
4	21.7 ± 1.3^{c}	19.9 ± 1.4^{c}	14.3 ± 1.3^{b}	15.5 ± 1.7^{c3}	0.001	NS
6	20.4 ± 1.2^{c}	17.5 ± 1.4^{a}	12.3 ± 0.9^{a}	14.2 ± 1.6^{c3}	0.001	NS
			L-[U-^{14}C]-leucine			
	(n=5)	(n=6)	(n=5)	(n=5)		
0	13.8 ± 0.7^{a}	12.0 ± 0.9^{a}	8.5 ± 1.0^{a}	6.7 ± 0.5^{a}	0.001	0.05
2	21.0 ± 1.2^{b}	24.6 ± 1.6^{b}	12.5 ± 1.7^{b}	11.1 ± 0.4^{b}	0.001	NS
4	18.9 ± 1.1^{c}	17.5 ± 1.2^{c}	13.1 ± 1.7^{b}	9.9 ± 0.5^{c}	0.001	NS
6	16.7 ± 1.5^{abc}	16.1 ± 1.4^{ac}	11.1 ± 0.9^{b}	7.9 ± 0.5^{d}	0.001	NS

[1] Significance of diet and rat effect by analysis of variance. [2] Means not charing a common character per group are significantly different ($P<0.05$). [3] These means of the trained LP group are not significantly different from the HP group.

Table 3.
Effects of diet and training on meal-induced changes in $^{14}CO_2$ output.

Diet...	High protein		Low protein		ANOVA (P)[1]	
Rats...	Control	Trained	Control	Trained	Diet	Rats
Time	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE		
			L-[1-^{14}C]-leucine			
	(n=7)	(n=5)	(n=4)	(n=7)		
0	13.0 ± 0.8[a2]	13.9 ± 1.4[a]	8.8 ± 0.6[a3]	10.1 ± 0.8[a3]	0.001	NS
2	29.3 ± 1.2[b]	28.2 ± 2.3[b]	26.7 ± 4.2[b]	29.4 ± 1.6[b]	NS	NS
4	27.0 ± 1.0[b]	27.4 ± 2.3[b]	25.9 ± 2.5[b]	29.1 ± 2.1[b]	NS	NS
6	26.7 ± 0.4[b]	23.1 ± 2.1[c]	26.0 ± 2.1[b]	29.0 ± 2.3[b]	NS	NS
			L-[U-^{14}C]-leucine			
	(n=5)	(n=4)	(n=5)	(n=6)		
0	8.9 ± 0.5[a]	8.0 ± 0.3[a]	5.2 ± 0.3[a3]	5.7 ± 0.4[a3]	0.001	NS
2	22.1 ± 1.2[b]	19.9 ± 0.8[b]	18.5 ± 1.3[b]	20.3 ± 2.0[b]	NS	NS
4	23.4 ± 1.4[b]	20.5 ± 1.6[b]	19.5 ± 1.8[b]	20.9 ± 1.6[b]	NS	NS
6	22.0 ± 1.4[b]	19.3 ± 1.7[b]	20.9 ± 1.3[b]	20.5 ± 2.2[b]	NS	NS

[1] Significance of diet and rat effect by analysis of variance. [2] Means not charing a common character per group are significantly different (P<0.05). [3] Significantly different from high protein diet (P<0.05).

DISCUSSION

Objectives

The main objective of the present study was to investigate the effect of training on leucine utilisation after exercise and feeding.

Numerous investigations have been performed on the effect of exercise on leucine metabolism. However, there is a wide variety in parameters, type of exercise, exercise duration and intensity, dietary composition, training status and especially the time of measurement. Therefore it is diffecult to establish the effect of exercise on amino acid

metabolism. It seems quite well demonstrated that leucine oxidation is increased during exercise (Rennie *et al.* 1981; White & Brooks, 1981; Hagg *et al.* 1982; Lemon *et al.* 1982; Wolfe *et al.* 1982; Lemon *et al.* 1985; Knapik *et al.* 1991). However, leucine oxidation has been found either unchanged (Tarnopolsky *et al.* 1991) or decreased (Devlin *et al.* 1990) after exercise.

This latter discrepancy can be explained by the effect of nutritional condition on leucine oxidation. Devlin *et al.* (1990) investigated the effect of bicycle exercise (75% $VO_{2,max}$; untill exhaustion) in overnight fasted humans and found leucine oxidation to be decreased by 30% in hour 3 after exercise. While Tarnopolsky *et al.* (1991) investigated the effect of resistance exercise in constantly fed humans and found leucine oxidation to be unchanged in hour 1 and hour 2 after exercise. In a previous study (**Chapter 2**) it was demonstrated that fasting improved the efficient use of leucine, while feeding decreased efficiency of utilisation. Therefore the effect of exercise on leucine oxidation might be masked by the effect of feeding. In the present study the effect of exercise was investigated in the early postabsorptive phase in order to exclude the acute effect of feeding.

The hypothesis of Millward & Rivers (1988) of the diurnal protein cycling, consists of postprandial protein gain and postabsorptive protein loss. In the mature animal these two components should be of equal size. For the growing animal there is some nett effect but the cycling nonetheless exists. This diurnal protein cycling is nutritionally sensitive, which is demonstrated by a higher amplitude of cycling with a higher level of dietary protein intake (Millward *et al.* 1991). The larger protein gain after feeding provides a larger amount of leucine for postabsorptive leucine oxidation. When a single bout of exercise causes an increase in leucine oxidation, leucine can be mobilised from the transient protein gain which accounts for this loss of leucine. However, when exercise is regularly repeated (training) it can be considered as an obligatory part of leucine requirement. It can be expected that diurnal protein metabolism shows some adaptation to exercise training: reduce losses due to exercise, increase feed intake or reduce losses during feed intake.

Exercise seemed to increase the postabsorptive protein loss (Viru, 1987), but training seemed to improve the nitrogen balance (Todd *et al.* 1984). Therefore training might either involve an increased postprandial protein gain and/or a decreased postabsorptive (postexercise) protein loss.

Effect of diet

The low protein diet resulted in a profound reduction in growth rate (**Table 1**) and probably in diurnal protein cycling. In the postabsorptive state the percentage leucine

oxidation was decreased by chronic protein restriction. The availability of leucine may well interfere with the adaptation to training.

Effect of exercise and/or stress

Especially in the first hour after exercise the output of radiolabelled carbondioxide was increased. Since the same amount of radiolabelled leucine is infused, the percentage leucine oxidation is increased. This observation is possibly confounded by an acute decrease of the labelled free leucine pool size, causing more label to be expired than is produced. However the plasma leucine concentration is significantly increased during this period for both diets, which suggests that there is even a larger leucine pool. Another problem may be the increased expiration of carbondioxide as a result of exercise. Bicarbonate recovery was not measured in this experiment. However, the increased expiration of carbondioxide was not considered a problem since during respiration measurements CO_2 production (data not shown) rapidly decreased after exercise to the pre-exercise level (within 15 min, including delay of the system). Thus it is concluded that leucine oxidation is significantly increased during 1 h after exercise and still elevated upto 5 h after exercise.

This surge in the percentage leucine oxidation was also found in rats subjected to only 10 min swimming (only trained LP group). This suggests that this response is not caused by 1 h endurance exercise, but by e.g. cleaning activity and cold stress. Also these 'stressed' rats showed an elevated percentage leucine oxidation upto 5 h after the 10 min swim.

Interaction between training and feeding

Feed intake per kg body weight was not different between control and trained rats, thus any increase in leucine oxidation during and after exercise should be compensated by an increased efficiency of leucine utilisation at some other time, e.g after feeding. Training however had no significant effect on the percentage leucine oxidation after feeding, when all groups were analysed together. Since acute protein supply increases the percentage of leucine oxidation by 2 to 3 fold for several hours, this is not surprising. However, the trained rats on the HP diet showed a significant lower percentage leucine oxidation at hour 6 *v.* hour 2 and 4 after a HP meal. In a previous study (**Chapter 1**) the same was found for untrained 18 h fasted rats. Since the present rats had been fasted for 30 h before the HP meal was consumed, training seems to improve the energy status of the animal. Another indication for an improved energy status is the significant decrease in total oxidation of leucine (UL) to CO_2 in the 6 h fasted rats (before exercise).

The trained LP group did not show an improvement in metabolic leucine utilisation, but this can be explained by the relative large amount of protein in the HP meal (1.05 g) compared to the normal daily intake (0.9 g). In fact, an even larger effect of training on metabolic leucine utilisation after a LP meal would be expected as is outlined in the next paragraph.

Interaction between training and exercise

When all groups were analysed together the effect of training on the percentage leucine oxidation after exercise was not significant. However, training decreased the percentage leucine oxidation after exercise for the HP group and increased it for the LP group. Rats conditioned on a LP diet are expected to have a smaller postprandial protein gain compared to rats conditioned on a HP diet. Therefore the percentage leucine oxidation after a single bout of exercise (control group) still reflects the protein status of the animal, which is illustrated by the large difference between the HP and LP group. While regularly repeated exercise (trained group) affects postabsorptive protein loss and presumably postprandial protein gain, which reduces the effect of chronic protein restriction on the percentage leucine oxidation. This is illustrated by the small difference between the HP and LP group, and can be considered as adaptation to training. Since training improved metabolic leucine utilisation in the HP group, both after feeding and after exercise, the higher percentage of leucine oxidation after exercise in the LP group should be fully compensated by improved utilisation of dietary leucine.

In conclusion, 1. chronic protein restriction improved metabolic leucine utilisation in the postabsorptive state, both before and after exercise, 2. training improved metabolic leucine utilisation in the high protein group both in the post-exercise and postprandial state, and 3. it is suggested that adaptation to training is especially relevant to the low protein diet.

GENERAL DISCUSSION

ON THE METHOD

The aim of the study was to identify and assess causes and objectives of indispensable amino acid losses and therefore the physiological and metabolic basis for their requirements.

There are many factors that cause amino acid oxidation. However, a common feature is the imbalance between amino acid supply and amino acid utilisation for protein synthesis.

Amino acids are supplied by an exogenous (digestion of dietary protein) or an endogenous source (breakdown of body protein). They are supplied to the free amino acid pool, also called the metabolic pool. From this pool amino acids can be used for body protein synthesis and as a precursor for other substances, but cannot be stored. Increased plasma and tissue levels of amino acids mainly increase oxidation of these amino acids or sometimes excretion in the urine. This is a very simple model of protein and amino acid metabolism.

When amino acid supply increases, amino acid utilisation has to change in accordance or the metabolic pool will expand. This is particularly true for the indispensable amino acids, like leucine, since they have a small metabolic pool relative to the rate at which the amino acids enter or leave the metabolic pool.

When the two rates are equal the metabolic pool size does not change. When additionally the rates do not change in size, there is a steady state and the rates can be measured by the isotope dilution method ('amino acid flux').

The amino acid flux can be measured by means of a constant radioactive isotope (labelled amino acid) infusion, and determination of the specific activity (enrichment for stable isotopes) of the amino acid in the metabolic pool. In isotope studies the metabolic pool is most often referred to as the precursor pool. The most accessable part of the precursor pool is blood plasma. In order to calculate the absolute value of

amino acid oxidation, the specific activity of waste products (e.g. expired CO_2 or urinary urea) has to be analysed and compared with the specific activity of the precursor pool.

The conditions required for the flux measurements cannot be met by 'normal' intermittent feeding and 'normal' diurnal changes in the physiological condition of humans and animals. In other words, the effect of acute changes in amino acid supply and utilisation cannot be investigated.

The aim of the present study was to find the factors that increase amino acid oxidation. This goal can be met by measuring the changes in the percentage amino acid oxidation as a result of protein nutrition and physiological conditions. The aim was not to measure the absolute values of amino acid oxidation in order to estimate amino acid requirements.

In the present study leucine was used because it is an indispensable (essential) amino acid. Therefore leucine is only derived from body protein or dietary protein. As an intact amino acid it is used for protein synthesis, and not for other biochemical processes. The rate of transamination of leucine is high (Cheng *et al.* 1985; Harper, 1985; Nissen & Haymond, 1986; Thompson *et al.* 1988). Therefore the irreversible step in the breakdown of the keto acid (α-ketoisocaproate, KIC) is the important control step. Decarboxylation of KIC is catalysed by the enzyme branched-chain keto acid dehydrogenase (BCKA-DH), which can be activated by dephosphorylation (Block *et al.* 1990). After the irreversible decarboxylation and the carbon skeleton is subsequently degraded to three acetyl-CoA molecules. Leucine is therefore called a ketogenic amino acid. Acetyl-CoA may either release CO_2 in the citric acid cycle, or can be used as a precursor for ketone bodies or lipogenesis.

Leucine oxidation studies depend on an isotopic approach, which introduces several problems:

1. Changes in pool size and specific activity.

The tracer infusion rate is constant, but the amount of tracer disappearing from the pool may change. When the free leucine pool is increasing or decreasing, the amount of tracer remaining in the pool may increase or decrease accordingly. This may temporarily cause an underestimate or overestimate of the percentage of leucine subjected to decarboxylation. After a change in the unlabelled leucine pool size, labelled leucine will move along it's own concentration gradient and restore the isotope equilibrium as set in the previous 'steady state' by the tracer infusion rate (Wolfe, 1984).

2. Sequestration of labelled C-skeleton.

When leucine is only ^{14}C-labelled in the carboxyl group, decarboxylation produces

$^{14}CO_2$ and problem 3 arises. As described above the C-skeleton is subsequently degraded to acetyl-CoA, which can be used for several processes (Meikle & Klain, 1972; Nissen *et al.* 1990; Wolfe & Jahoor, 1990; Yoshida *et al.* 1991). The label is distributed among all intermediates, and the recovery of label decreases. This is particularly relevant to universally labelled leucine, and underestimates the level of oxidation.

Although it would be expected that this non-protein label retention is determined by the energy status (acetyl-CoA pool), from the present study it seems to be related to oversupply of the amino acid relative to the metabolic status of the animal (**Chapter 1**). When universally labelled leucine is completely oxidised to $^{14}CO_2$ the next problem arises.

3. Recovery of labelled CO_2.

The production of labelled carbon dioxide is higher than the expiration of $^{14}CO_2$. The recovery of label from the body is less than 100% and therefore the level of oxidation is probably underestimated. This problem has often been studied with labelled bicarbonate. Reported levels of label recovery vary, but on average were 80% for larger animals and humans (Allsop *et al.* 1978; Hoerr *et al.* 1989) and upto 96% for the rat (Yagi & Walser, 1990).

Chapter 1 shows that the first response to meal ingestion is a transient increase in $^{14}CO_2$ output (0 to 1.5 h after onset of meal), even when a protein free meal is consumed. It has been demonstrated that the leucine flux (protein breakdown) is decreased upon carbohydrate intake, which restricts substrate availability for protein synthesis and acutely reduces the leucine pool size (Nissen & Haymond, 1986; Nair *et al.* 1987a). It seems unlikely that the percentage of labelled leucine subjected to decarboxylation is increased in this condition, but it cannot be excluded.

However, the measurements indicate the time scale upon which these changes occur. The first response may indicate a decrease in protein breakdown. This would be an ideal first response to meal ingestion, since it allows to use available protein synthesis capacity for deposition of dietary amino acids.

RELATIONSHIP BETWEEN ABSOLUTE AND RELATIVE MEASURE

From literature values, a strong relationship (r=0.92) was found between the absolute measure (μmol/kg.h) and the relative measure (% of flux) of leucine decarboxylation. This relationship indicates that in the lower range of the percentage leucine decarboxylation (between 5% and 25%), there is an almost linear relationship.

However, above 25% the absolute values of leucine decarboxylation increase drastically, with only minimal increases in the percentage leucine decarboxylation. This suggests that any significant increase in the percentage leucine decarboxylation from 30% to 35%, as after a high protein meal in mature rats (**Chapter 1**), might constitute a profound increase in the absolute value of leucine decarboxylation. Thus in this range any increase in the utilisation efficiency would largely increase the amount of dietary leucine that is deposited (in the fed state), or reduce the amount of leucine loss (in the postabsorptive state).

EFFECT OF GROWTH AND PREGNANCY

Since the recognition of the dynamic state of body protein, the physiological meaning of protein turnover has been considered (Waterlow *et al.* 1978). Protein turnover is quanlitatively important for maintaining functional body proteins. Recently Millward & Rivers (1988) have extended this classical idea of protein turnover, with diurnal protein cycling. Diurnal protein cycling consists of postprandial protein gain (dietary amino acid deposition) and postabsorptive protein loss (amino acid oxidation). This cycling of body protein is quantitatively important, and can be regarded as endogenous feeding. Both growing and mature animals deposit protein after feeding and use it during fasting, with the difference that growing animals show a net gain of body protein. There are essentially two opportunities for growth, either dietary protein is used more efficiently for protein deposition or endogenous protein is reused more efficiently for protein synthesis.

Chapter 1 shows that the utilisation efficiency in the postabsorptive state was higher for growing animals on either diet. Ingestion of a meal resulted in a fast increase in the percentage leucine oxidation. Two to four hours after the low protein meal, but only two hours after a high protein meal, the utilisation efficiency seemed higher in the growing animals.

Thus growing animals have a significantly improved utilisation efficiency in the early postprandial phase. The non-oxidative leucine disposal (protein synthesis) was about 70% for mature animals and 75% for growing animals. This difference contributes to growth, but mainly indicates that in the postprandial state both the growing and mature animal direct the larger part of leucine to body protein. This is in line with the diurnal protein cycling concept.

Chapter 3 shows with oral tracer administration an improved utilisation efficiency for growing versus mature animals after a high protein meal. The two methods are not

entirely comparable, but the difference between growing and mature rats seemed to be larger with intravenous tracer administration. This suggests that the difference in the percentage leucine oxidation is due to differences in peripheral tissues (e.g. muscle) and not splanchnic tissues.

Chapter 4 shows that pregnancy (mature growing animals) improves the postabsorptive utilisation efficiency, and tends to decrease the percentage leucine oxidation after a low protein meal, but not after a high protein meal.

Thus there is evidence for both postabsorptive and postprandial improvement of metabolic leucine utilisation during growth and pregnancy.

The main difference between growing and mature animals seems to be the ability of growing rats to increase muscle protein synthesis upon feeding (Garlick *et al.* 1983; Preedy & Garlick, 1986; Garlick & Grant, 1988), as opposed to mature rats (Baillie *et al.* 1988; Garlick *et al.* 1989a; Garlick *et al.* 1990) and adult humans (Wernerman *et al.* 1985). While liver protein synthesis does not seem to change (Garlick *et al.* 1973), it might also increase in the growing rat (Simon & Bergner, 1983; Simon, 1989).

EFFECT OF ACUTE AND CHRONIC PROTEIN SUPPLY

It is well recognised that chronic protein restriction reduces amino acid oxidation (**General Introduction, Table 1**; Young & Marchini, 1990). This can be achieved by an increased reutilisation of amino acids for protein synthesis, due to a reduction in protein turnover and/or a reduction in oxidative activity. Although the increased efficiency will be regarded as beneficial, the decrease in protein turnover might reduce the ability for adaptive responses.

Voit observed in 1867 that, when dogs were changed from a low to a high protein intake, nitrogen was retained; after changing from a high to a low protein intake, nitrogen was lost from the body (Munro, 1964). This indicates a slow adjustment in nitrogen metabolism to a change in protein intake.

Diurnal protein cycling increases with increased protein intake (Millward & Rivers, 1988). When protein intake is acutely changed to a low level, the postprandial protein gain decreases but initially the postabsorptive oxidative losses continue and result in a nitrogen loss (Millward *et al.* 1990; Price *et al.* 1990). This indicates a delayed response in postabsorptive amino acid losses.

Chapter 1 and 6 show that the utilisation efficiency in the postabsorptive state is improved by chronic protein restriction. It is also shown that the efficiency decreases immediately upon ingestion of a meal. **Chapter 1** shows that the level of leucine

oxidation is determined by the protein content of the meal. This indicates a very fast response of leucine oxidation to acute protein supply, while postabsorptive amino acid oxidation and nitrogen excretion have been shown to be delayed. It also indicates that amino acid losses increase with increased protein intake, which results in a non-linear response of nitrogen balance to protein intake (Munro, 1985). Studies on enzyme kinetics have shown that in meal fed rats, BCKA-DH is inactivated in the postabsorptive state and quickly reactivated upon feeding (Block *et al.* 1990). This mechanism supports a fast response of leucine oxidation to feeding.

In humans 40% of dietary amino acids was oxidised within 4 hours after a mixed meal (Elia *et al.* 1989). In dogs 57% of dietary amino acids was oxidised within 12 hours after a protein meal (Elwyn, 1970). Therefore this response of leucine oxidation upon feeding probably also holds for other amino acids.

In mature humans the main response to feeding is a decrease in protein breakdown (Garlick *et al.* 1990), whereas an increase in protein synthesis has been suggested (Millward *et al.* 1991). In dogs it has been shown that the decrease in protein breakdown is independent of the dietary amino acid composition or content (Nissen & Haymond, 1986). It is suggested that mainly glucose decreases protein breakdown via stimulation of insulin action (Nair *et al.* 1987a, 1987b; Pacy *et al.* 1989, 1991).

Several studies have indicated that feeding reduces liver protein breakdown substantially (Garlick *et al.* 1973; Millward *et al.* 1974; Conde & Scornik, 1976; Waterlow *et al.* 1978; Khairallah *et al.* 1985; Mortimore & Pösö, 1987). Especially leucine is a potent inhibitor of liver protein breakdown (Pösö & Mortimore, 1985; Mortimore & Pösö, 1987). This provides liver protein synthesis capacity for deposition of dietary amino acids. After feeding the liver protein content may increase by about 25% (Garlick *et al.* 1973; Millward *et al.* 1974; Waterlow *et al.* 1978).

The normal responses to fasting and protein deprivation is that the liver acutely (1-2 days) looses protein, while muscle tissue shows a slower response (2-5 days) (Addis *et al.* 1936a, 1936b; Millward *et al.* 1974; Garlick *et al.* 1975), and after refeeding the liver regains protein very fast (Addis *et al.* 1936c; Conde & Scornik, 1976; Hutson & Mortimore, 1982). This suggests that mainly the liver, and possibly the gut, shows diurnal protein cycling (Simon, 1989). Protein lost from liver and gut can be used as endogenous amino acid supply ('endogenous feeding') for proteins with a slower turnover rate, like muscle.

In **Chapter 5** the breakdown of newly synthesized protein probably causes a large recycling of radioactive label. The level of protein breakdown after feeding is in fact the level of protein cycling and 'endogenous feeding'. The higher postabsorptive level of leucine oxidation suggests a higher level of protein cycling when daily intake is

devided in smaller portions.
It is suggested that mainly muscle tissue benifits from smaller meals, while liver and gut have to buffer the larger meals. This intermediairy protein deposition provides a protein store for amino acids that would otherwise be oxidised.

EFFECT OF FASTING AND FEEDING

Leucine oxidation has been shown to be lower in the postabsorptive (overnight fasted) state compared to the fed state (Garlick *et al.* 1980; Motil *et al.* 1981b; Clugston & Garlick, 1982; Rennie *et al.* 1982; Hoffer *et al.* 1985; Melville *et al.* 1989). However, prolonged fasting (longer than overnight fasting) was found to increase leucine oxidation in rats (Meikle & Klain, 1972; Sketcher *et al.* 1974; Vazquez *et al.* 1986) and humans (Tsalikian *et al.* 1984; Nair *et al.* 1987d; Jensen *et al.* 1988; Knapik *et al.* 1991).
Chapter 2 shows that the utilisation efficiency decreases with an increased period of fasting. With intravenous tracer infusion fasting had no effect on the postprandial utilisation efficiency at 4 h after the meal. However 6 hours after the meal the percentage leucine oxidation remains slightly higher when animals had been fasted longer than overnight. But when tracer was provided with the meal, there was a profound negative effect of previous fasting on metabolic leucine utilisation.
After an overnight fast the comparison between oral and intravenous tracer administration showed that dietary leucine is more efficiently used by the intestinal mucosa than by the peripheral tissues. This can be explained by the preferential use of intraluminal (dietary) leucine for protein synthesis in the intestinal mucosa (Hirshfield & Kern, 1969; Alpers, 1972). However, when rats were fasted longer than overnight dietary leucine was much less efficiently used by the intestinal mucosa than after an overnight fast. This was not observed with intravenous tracer infusion, which suggests that peripheral tissues are not involved.
This might be related to the high glutamine uptake of the gut, which is released by muscle (Wahren *et al.* 1976). Although this release is continuous before and after feeding (Wahren *et al.* 1976; Elia *et al.* 1989), prolonged fasting might decrease the glutamine supply to the gut or increase the need for glutamine. Transamination of leucine might be increased in order to increase the glutamine concentration of the gut. Leucine can provide nitrogen to α-ketoglutarate to form glutamine and α-ketoisocaproate can be released and taken up by the liver for decarboxylation.
It has been demonstrated that the gut has substantial BCAAT activity (Mimura *et al.*

1968; Abumrad *et al.* 1989), while the liver has a high BCKA-DH activity (Hutson, 1989). In humans splanchnic BCAA release accounted for 50% of dietary intake (Wahren *et al.* 1976). It has been demonstrated that large amounts (upto 37%) of dietary leucine are metabolised in the splanchnic tissues before entering the systemic circulation, this is called the 'first pass effect' (Cortiella *et al.* 1988; Imura *et al.* 1988; Istfan *et al.* 1988; Melville *et al.* 1989; Yu *et al.* 1990; Hoerr *et al.* 1991). It has been suggested that liver protein synthesis constitutes only a very small part of splanchnic leucine removal (Istfan *et al.* 1988). However, that would not be in line with postprandial liver protein deposition.

It is suggested that the energy status of the intestine is involved in the utilisation of dietary leucine. In the postabsorptive state the activity of BCKA-DH shows a better correlation with the tissue ATP level than with plasma and tissue levels of BCAA or BCKA (Kasperek, 1989).

EFFECT OF EXERCISE AND TRAINING

Exercise has been shown to increase leucine oxidation during exercise (Rennie *et al.* 1981; White & Brooks, 1981; Hagg *et al.* 1982; Wolfe *et al.* 1982; Knapik *et al.* 1991). The effect of exercise on leucine oxidation during the recovery period is unclear.

Chapter 6 shows that exercise decreases the (re)utilisation efficiency both acutely and for several hours after exercise. Adaptation to training and chronic protein restriction interact with respect to leucine oxidation. This interaction is probably on the level of diurnal protein cycling. Protein gain after feeding has to account for any postabsorptive amino acid loss. When this postabsorptive loss is increased by a single bout of exercise, or in general by any form of 'metabolic stress', this will increase protein loss. This may be the signal for increased protein gain with the next feeding, although it might also take several days or even weeks before there is significant adaptation in protein gain. Training improved metabolic leucine utilisation at 6 hours after a high protein meal, and seemed to decrease the response to a bout of exercise. Rats conditioned on a low protein diet however increased the response of leucine oxidation to exercise, which can only be explained by a larger improvement in metabolic leucine utilisation after a low protein meal. This was in line with observed influences of growth and pregnancy, where also the low protein meal benifits more from a physiological adaptation but was also more in need of this adaptation.

CONCLUDING REMARK

Metabolic leucine utilisation as a relative measure can be a useful tool to investigate the effect of different nutritional and/or physiological conditions on protein and amino acid metabolism. The relative measure provides information about the utilisation efficiency, which may change acutely upon e.g. feeding or exercise.

Table 1.
Effects of dietary protein and physiological condition on postprandial metabolic leucine utilisation.

Factor	Time after meal	Effect	Mechanism?
1. meal	hour 1	OX↑?	PB↓
2. acute protein supply	hour 2 tot 6	OX↑	PI↑
3. growth/pregnancy	hour 2 to 4	OX↓	PS↑
4. chronic protein restriction	hour 4 to 6	OX↓	PB↓?
5. previous fasting	hour 6	OX↑	PB↑?
6. training	hour 6	OX↓	PS↑?

OX, leucine oxidation; PB, protein breakdown; PS, protein synthesis; PI, protein intake

The results show that various physiological conditions influence postprandial leucine utilisation, although the level of acute protein supply is most important (**Table 1**). This indicates that a physiological feeding strategy should be concerned with various aspects of normal diurnal variation in protein and amino acid metabolism. The physiological limitations to dietary amino acid utilisation need special consideration in clinical and animal nutrition.

FUTURE PERSPECTIVES

The main conclusion is that dietary protein in itself increases leucine oxidation and therefore decreases the utilisation efficiency. This is both relevant to inefficient use of dietary protein supply, and increased loss of endogenous leucine. However this loss cannot be identified as an endogenous or physiological requirement, but is rather a nutritional requirement. This suggests that it is less useful to try and determine the requirements for protein and/or amino acids as such than considering the aptitude of

dietary supply in relation to the physiological condition.

Not only the balance between dietary input and products is important, but rather the metabolism in between. The balance is just the situation at one moment. It is more important for what purpose the dietary amino acids are used, whether the objective is of an obligatory, a reserve or wasteful nature. But this depends on the views or the goals in question.

A high level of protein deposition after feeding is obligatory since otherwise dietary amino acids are wasted immediately, and cannot be used in the postabsorptive state. The efficiency of dietary amino acid utilisation is initially set by the level of postprandial oxidation. Thereafter the efficiency can only become less by oxidation of amino acids available from what has now become endogenous protein.

One of the most important questions is how to maintain a high level of protein deposition, but with a high efficiency. A complication of this matter is the more specific site of utilisation of dietary protein. A large retention as such does not indicate whether the splanchnic tissues benefit more or less than peripheral tissues, like muscle and reproductive tissues. On the other hand the latter tissues might benefit later from the storage function the former tissues have. In simple terms: it's available, but what to do with it?

Most often the importance of muscle and reproductive tissue has been emphasized. Particularly when muscle tissue is not maintained properly, loss of lean tissue (e.g. after physical trauma) or a reduced growth rate (e.g. bacterial infection of production animal) is likely. Patients require longer hospital treatment and production animals remain eating but produce less. We don't want this because of economical and ethical reasons, but it is physiologically sound. First priority is to keep the organism alive. Muscle tissue is more or less a bulk protein, which can be reduced substantially. And less favourable circumstances cause stunted growth and reduce reproduction, but both may be biologically very important.

Although all proteins may be regarded as functional, one might be more essential than another. Reduction of gut function might easily break down the defence against bacteria etc., with bacterial translocation and shock as a consequence. Reduction of liver function decreases detoxification of dietary or metabolic compounds and production of e.g. the so-called acute phase proteins.

This emphasizes the need for constant maintenance of proteins, firstly with the aim to remain healthy and secondly to grow or produce. Therefore it is necessary to understand the mechanisms involved and the relationships between different aspects.

Although protein deposition in the splanchnic tissues seems to function as a buffer for peripheral tissues, this might also play a very important role for protein turnover in

these tissues. This also provides a reserve function. In any case where a metabolic perturbation or metabolic stress increases the requirement, amino acids can be supplied from this reserve. If no perturbation or stress occurs, most amino acids will be wasted afterwards, but might have served an obligatory function initially.

It is therefore suggested that efforts aimed at improving amino acid utilisation should be focussed on increasing the postprandial protein deposition. The development of a physiological feeding strategy might both reduce health hazards (by increasing the reserve capacity) and reduce dietary and therefore financial input (by increasing the utilisation efficiency). In practice, in human nutrition reduction of health hazards for long term health might be preferred and in animal nutrition the economical aspect and environmental considerations might be more important, but one doesn't go without the other.

Peter Weijs
Wageningen, November 16, 1992

REFERENCES

Abumrad, N.N., McRae, J.R. & Lacy, W.W. (1984) Inadequacy of leucine isotopic methods for measurements of whole body protein turnover in vivo. *Clinical Research* **32**, 387A.

Abumrad, N.N., Williams, P., Frexes-Steed, M., Geer, R., Flakoll, P., Cersosimo, E., Brown, L.L., Melki, I., Bulus, N., Hourani, H., Hubbard, M. & Ghishan, F. (1989) Inter-organ metabolism of amino acids in vivo. *Diabetes/Metabolism Reviews* **5**, 213-226.

Addis, T., Poo, L.J. & Lew, W. (1936a) The quantities of protein lost by the various organs and tissues of the body during a fast. *Journal of Biological Chemistry* **115**, 111-116.

Addis, T., Poo, L.J. & Lew, W. (1936b) Protein loss from liver during a two day fast. *Journal of Biological Chemistry* **115**, 117-118.

Addis, T., Poo, L.J. & Lew, W. (1936c) The rate of protein formation in the organs and tissues of the body. I. After casein refeeding. *Journal of Biological Chemistry* **116**, 343-352.

Allsop, J.R., Wolfe, R.R. & Burke, J.F. (1978) Tracer priming the bicarbonate pool. *Journal of Applied Physiology* **45**, 137-139.

Alpers, D.H. (1972) Protein synthesis in intestinal mucosa: the effect of route of administration of precursor amino acids. *Journal of Clinical Investigation* **51**, 167-173.

Baillie, A.G.S., Maltin, C.A. & Garlick, P.J. (1988) The effect of fasting and insulin infusion on muscle protein synthesis in immature and adult rats. *Proceedings of the Nutrition Society* **47**, 114A.

Ball, R.O. & Bayley, H.S. (1985) Time course of the total and radioactive carbon dioxide production by piglets receiving dietary [^{14}C]phenylalanine. *Canadian Journal of Physiology and Pharmacology* **63**, 1170-1174.

Batterham, E.S. (1974) The effect of frequency of feeding on the utilization of free lysine by growing pigs. *British Journal of Nutrition* **31**, 237-242.

Batterham, E.S. & O'Neill, G.H. (1978) The effect of frequency of feeding on the response by growing pigs to supplements of free lysine. *British Journal of Nutrition* **39**, 265-270.

Batterham, E.S. & Bayley, H.S. (1989) Effect of frequency of feeding of diets containing free or protein-bound lysine on the oxidation of [^{14}C]lysine or [^{14}C]phenylalanine by growing pigs. *British Journal of Nutrition* **62**, 647-655.

Beach, E.F. (1948) Proteins in nutrition (Historical). In: *Proteins and Amino Acids in Nutrition*, pp. 1-45 [M. Sahyun, editor]. New York: Reinhold Publishing Corporation.

Beaton, G.H., Beare, J., Ryu, M.H. & McHenry, E.W. (1954) Protein metabolism in the pregnant rat. *Journal of Nutrition* **54**, 291-304.

Bender, D.A. (1985) *Amino Acid Metabolism*, 2nd ed., pp. 175-187. Chichester: John Wiley & Sons.

Bennet, W.M., Connacher, A.A., Scrimgeour, C.M., Smith, K. & Rennie, M.J. (1989) Increase in anterior tibialis muscle protein synthesis in healthy man during mixed amino acid infusion: studies of incorporation of [1-^{13}C]leucine. *Clinical Science* **76**, 447-454.

Bennet, W.M., Connacher, A.A., Smith, K., Jung, R.T. & Rennie, M.J. (1990) Inability to stimulate skeletal muscle or whole body protein synthesis in type 1 (insulin-dependent) diabetic patients by insulin-plus-glucose amino acid infusion: studies of incorporation and turnover of tracer L-[1-^{13}C]leucine. *Diabetologia* **33**, 43-51.

Bennet, W.M., Connacher, A.A., Jung, R.T., Stehle, P. & Rennie, M.J. (1991) Effects of insulin and

amino acids on leg protein turnover in IDDM patients. *Diabetes* **40**, 499-508.

Birt, D.F. & Clark, H.E. (1976) Free amino acid patterns and the distribution of ^{14}C from [U-^{14}C]-L-Leucine, [U-^{14}C]-L-Lysine, and [U-^{14}C]-L-Alanine in the tissues of young adult rats. *Journal of Nutrition* **106**, 597-605.

Block, K.P., Aftring, R.P. & Buse, M.G. (1990) Regulation of rat liver branched-chain α-keto acid dehydrogenase activity by meal frequency and dietary protein. *Journal of Nutrition* **120**, 793-799.

Brookes, I.M., Owens, F.N. & Garrigus, U.S. (1972) Influence of amino acid level in the diet upon amino acid oxidation by the rat. *Journal of Nutrition* **102**, 27-36.

Cahill, G.F. (1970) Starvation in man. *New England Journal of Medicine* **282**, 668-675.

Cahill, G.F. & Aoki, T.T. (1980) Partial and total starvation. In: *Assessment of energy metabolism in health and disease*, pp 129-134 [J.M. Kinney, editor]. Columbus, Ohio: Ross Laboratories.

Cathcart, E.P. (1925) The influence of muscle work on protein metabolism. *Physiological Reviews* **5**, 225-243.

Clugston, G.A. & Garlick, P.J. (1982) The response of protein and energy metabolism to food intake in lean and obese man. *Human Nutrition: Clinical Nutrition* **36C**, 57-70.

Cohn, C., Joseph, D., Bell, L. & Oler, A. (1963) Feeding frequency and protein metabolism. *American Journal of Physiology* **205**, 71-78.

Conde, R.D. & Scornik, O.A. (1976) Role of protein degradation in the growth of livers after a nutritional shift. *Biochemical Journal* **158**, 385-390.

Cortiella, J., Matthews, D.E., Hoerr, R.A., Bier, D.M. & Young, V.R. (1988) Leucine kinetics at graded intakes in young men: quantitative fate of dietary leucine. *American Journal of Clinical Nutrition* **48**, 998-1009.

Cuthbertson, D.P. (1954) Interrelationship of metabolic changes consequent to injury. *British Medical Bulletin* **10**, 33-37.

Cuthbertson, D.P., McGirr, J.L. & Munro, H.N. (1937) CCLXXXII. A study of the effect of overfeeding on the protein metabolism of man. IV. The effect of muscular work at different levels of energy intake, with particular reference to the timing of the work in relation to the taking of food. *Biochemical Journal* **31**, 2293-2305.

Devlin, J.T., Brodsky, I., Scrimgeour, A., Fuller, S. & Bier, D.M. (1990) Amino acid metabolism after intense exercise. *American Journal of Physiology* **258**, E249-E255.

Elia, M., Folmer, P., Schlatmann, A., Goren, A. & Austin, S. (1989) Amino acid metabolism in muscle and in the whole body of man before and after ingestion of a single mixed meal. *American Journal of Clinical Nutrition* **49**, 1203-1210.

Elwyn, D.H. (1970) The role of the liver in regulation of amino acid and protein metabolism. In: *Mammalian Protein Metabolism*, volume IV, pp. 523-557 [H.N. Munro, editor]. New York and London: Academic Press.

Erasmus (around 1615) *Cort Verhael van het leven Erasmi van Rotterdam zelfs van hem beschreven*. Rotterdam: Matthijs Bastiaensen.

Essen, P. (1991) *Muscle Protein Synthesis under Anesthesia and Surgery. Application of the Flooding Dose Method to Man*. Thesis. Stockholm: Karolinska Institute.

FAO/WHO/UNU (1985) *Energy and Protein Requirements*. Report of a joint FAO/WHO/UNU Expert Consultation. Technical Report Series 724. Geneva: World Health Organization.

FAO/WHO (1990) *Protein Quality Evaluation*. Report of a joint FAO/WHO Expert Consultation.

Rome: Food and Agricultural Organization of the United Nations.

Felig, P. (1975) Amino acid metabolism. *Annual Review of Biochemistry* **44**, 933-955.

Felig, P., Owen, O.E., Wahren, J. & Cahill Jr., G.F. (1969) Amino acid metabolism during prolonged starvation. *Journal of Clinical Investigation* **48**, 584-594.

Folin, O. (1905) A theory of protein metabolism. *American Journal of Physiology* **13**, 117-138.

Fukagawa, N.K., Minaker, K.L., Young, V.R., Matthews, D.E., Bier, D.M. & Rowe, J.W. (1989) Leucine metabolism in aging humans: effect of insulin and substrate availability. *American Journal of Physiology* **256**, E288-E294.

Garlick, P.J., Millward, D.J. & James, W.P.T. (1973) The diurnal response of muscle and liver protein synthesis *in vivo* in meal-fed rats. *Biochemical Journal* **136**, 935-945.

Garlick, P.J., Millward, D.J., James, W.P.T. & Waterlow, J.C. (1975) The effect of protein deprivation and starvation on the rate of protein synthesis in tissues of the rat. *Biochimica et Biophysica Acta* **414**, 71-84.

Garlick, P.J., Clugston, G.A., Swick, R.W. & Waterlow, J.C. (1980) Diurnal pattern of protein and energy metabolism in man. *American Journal of Clinical Nutrition* **33**, 1983-1986.

Garlick, P.J., McNurlan, M.A. & Preedy, V.R. (1980) A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [^{3}H]phenylalanine. *Biochemical Journal* **192**, 719-723.

Garlick, P.J., Fern, M. & Preedy, V.R. (1983) The effect of insulin infusion and food intake on muscle protein synthesis in postabsorptive rats. *Biochemical Journal* **210**, 669-676.

Garlick, P.J. & Grant, I. (1988) Amino acid infusion increases the sensitivity of muscle protein synthesis *in vivo* to insulin. *Biochemical Journal* **254**, 579-584.

Garlick, P.J., Maltin, C.A., Baillie, A.G.S., Delday, M.I. & Grubb, D.A. (1989a) Fiber-type composition of nine rat muscles. II. Relationship to protein turnover. *American Journal of Physiology* **257**, E828-E832.

Garlick, P.J., Wernerman, J., McNurlan, M.A., Essen, P., Lobley, G.E., Milne, E., Calder, G.A. & Vinnars, E. (1989b) Measurement of the rate of protein synthesis in muscle of postabsorptive young men by injection of a 'flooding dose' of [1-^{13}C]leucine. *Clinical Science* **77**, 329-336.

Garlick, P.J., Wernerman, J., McNurlan, M.A. & Essen, P. (1990) What is the normal response of protein turnover to nutrient supply? *Clinical Nutrition* **9**, 294-296.

Gontzea, I., Sutzescu, R. & Dumitrache, S. (1975) The influence of adaptation to physical effort on nitrogen balance in man. *Nutrition Reports International* **11**, 231-236.

Goto, M., Shinno, H. & Ichihara, A. (1977) Isozyme patterns of branched-chain amino acid transaminase in human tissues and tumors. *Gann* **68**, 663-667.

Griggs, R.C., Jozefowicz, R., Kingston, W., Nair, K.S., Herr, B.E. & Halliday, D. (1990) Mechanism of muscle wasting in myotonic dystrophy. *Annals of Neurology* **27**, 505-512.

Hagg, S.A., Morse, E.L. & Adibi, S.A. (1982) Effect of exercise on rates of oxidation, turnover, and plasma clearance of leucine in human subjects. *American Journal of Physiology* **242**, E407-E410.

Harper, A.E. (1986) Enzymatic basis for adaptive changes in amino acid metabolism. In: *Proceedings of the XIII International Congress of Nutrition*, pp. 409-414 [T.G. Taylor and N.K. Jenkins, editors]. London, Paris: John Libbey.

Harper, A.E. & Benjamin, E. (1984) Relationship between intake and rate of oxidation of leucine and α-ketoisocaproate in vivo in the rat. *Journal of Nutrition* **114**, 431-440.

Harris, R.A., Powell, S.M., Paxton, R., Gillim, S.E. & Nagae, H. (1985) Physiological covalent regulation of rat liver branched-chain α-ketoacid dehydrogenase. *Archives of Biochemistry and Biophysics* **243**, 542-555.

Hegsted, D.M. (1976). Balance studies. *Journal of Nutrition* **106**, 307-311.

Henderson, S.A., Black, A.L. & Brooks, G.A. (1985) Leucine turnover and oxidation in trained rats during exercise. *American Journal of Physiology* **249**, E137-E144.

Heymsfield, S.B., Casper, K. & Grossman, G.D. (1987) Bioenergetic and metabolic response to continuous v intermittent nasoenteric feeding. *Metabolism* **36**, 570-575.

Hirschfield, J.S. & Kern Jr., F. (1969) Protein starvation and the small intestine. III. Incorporation of orally and intraperitoneally administered L-Leucine 4,5-^{3}H into intestinal mucosal protein of protein-deprived rats. *Journal of Clinical Investigation* **48**, 1224-1229.

Hoerr, R.A., Yu, Y.-M., Wagner, D.A., Burke, J.F. & Young, V.R. (1989) Recovery of ^{13}C in breath from $NaH^{13}CO_3$ infused by gut and vein: effect of feeding. *American Journal of Physiology* **257**, E426-E438.

Hoerr, R.A., Matthews, D.E., Bier, D.M. & Young, V.R. (1991) Leucine kinetics from [$^{2}H_3$]- and [^{13}C]leucine infused simultaneously by gut and vein. *American Journal of Physiology* **260**, E111-E117.

Hoffer, L.J., Yang, R.D., Matthews, D.E., Bistrian, B.R., Bier, D.M. & Young, V.R. (1985) Effects of meal consumption on whole body leucine and alanine kinetics in young adult men. *British Journal of Nutrition* **53**, 31-38.

Hutson, N.J. & Mortimore, G.E. (1982) Suppression of cytoplasmic protein uptake by lysosomes as the mechanism of protein regain in livers of starved-refed mice. *Journal of Biological Chemistry* **257**, 9548-9554.

Hutson, S.M. (1989) Regulation of substrate availability for the branched-chain α-keto acid dehydrogenase enzyme complex. *Annals of the New York Academy of Sciences* **573**, 230-239.

Imura, K., Shiota, T., Swain, L.M. & Walser, M. (1988) Utilization for protein synthesis of 2-ketoisocaproate relative to utilization of leucine, as estimated from exhalation of labelled CO_2. *Clinical Science* **75**, 301-307.

Istfan, N.W., Ling, P.R., Bistrian, B.R. & Blackburn, G.L. (1988) Systemic exchangeability of enteral leucine: relationship to plasma flux. *American Journal of Physiology* **254**, R688-R698.

Jahoor, F., Shangraw, R.E., Miyoshi, H., Wallfish, H., Herndon, D.N. & Wolfe, R.R. (1989) Role of insulin and glucose oxidation in mediating the protein catabolism of burns and sepsis. *American Journal of Physiology* **257**, E323-E331.

Jensen, M.D., Miles, J.M., Gerich, J.E., Cryer, P.E. & Haymond, M.W. (1988) Preservation of insulin effects on glucose production and proteolysis during fasting. *American Journal of Physiology* **254**, E700-E707.

Jungas, R.L., Halperin, M.L. & Brosnan, J.T. (1992) Quantitative analysis of amino acid oxidation and related gluconeogenesis in humans. *Physiological Reviews* **72**, 419-448.

Kasperek, G.J. (1989) Regulation of branched-chain 2-oxo acid dehydrogenase activity during exercise. *American Journal of Physiology* **256**, E186-E190.

Khairallah, E.A., Bartolone, J., Brown, P., Bruno, M.K., Makowski, G. & Wood, S. (1985) Does glutathione play a role in regulating intracellular proteolysis? In: *Intracellular Protein Catabolism*, pp 373-383 [E.A. Khairallah, J.S. Bond and J.W.C. Bird, editors]. New York: Alan

R. Liss.

Kim, K.I., Elliot, J.I. & Bayley, H.S. (1983) Oxidation of an indicator amino acid by young pigs receiving diets with varying levels of lysine or threonine, and an assessment of amino acid requirements. *British Journal of Nutrition* **50**, 391-399.

Knapik, J., Meredith, C., Jones, B., Fielding, R., Young, V. & Evans, W. (1991) Leucine metabolism during fasting and exercise. *Journal of Applied Physiology* **70**, 43-47.

Layman, D.K. & Wolfe, R.R. (1987) Sample site selection for tracer studies applying a unidirectional circulatory approach. *American Journal of Physiology* **253**, E173-E178.

Lemon, P.W.R., Nagle, F.J., Mullin, J.P. & Benevenga, N.J. (1982) In vivo leucine oxidation at rest and during two intensities of exercise. *Journal of Applied Physiology* **53**, 947-954.

Lemon, P.W.R., Benevenga, N.J., Mullin, J.P. & Nagle, F.J. (1985) Effect of daily exercise and food intake on leucine oxidation. *Biochemical Medicine* **33**, 67-76.

Ling, P.R., Bistrian, B.R., Blackburn, G.L. & Istfan, N. (1987) Effect of fetal growth on maternal protein metabolism in postabsorptive rat. *American Journal of Physiology* **252**, E380-E390.

Ling, P.R., Bistrian, B.R., Blackburn, G.L. & Istfan, N. (1989) Effect of continuous feeding on maternal protein metabolism and fetal growth in the rat. *American Journal of Physiology* **256**, E852-E862.

Livesey, G. & Lund, P. (1980) Enzymic determination of branched-chain amino acids and 2-oxoacids in rat tissues. *Biochemical Journal* **188**, 705-713.

Matthews, D.E., Schwarz, H.P., Yang, R.D., Motil, K.J., Young, V.R. & Bier, D.M. (1982) Relationship of plasma leucine and α-ketoisocaproate during a L-[1-^{13}C]Leucine infusion in man: a method for measuring human intracellular leucine tracer enrichment. *Metabolism* **31**, 1105-1112.

Mayel-Afshar, S., Grimble, R.F. & Taylor, T.G. (1981) Tyrosine oxidation during pregnancy in normal and protein deficient rats. *Proceedings of the Nutrition Society* **40**, 36A.

Mayel-Afshar, S. & Grimble, R.F. (1982) Tyrosine oxidation and protein turnover in maternal tissues and the fetus during pregnancy in rats. *Biochimica et Biophysica Acta* **716**, 201-207.

Mayel-Afshar, S. & Grimble, R.F. (1983) Changes in protein turnover during gestation in the foetuses, placentas, liver, muscle and whole body of rats given a low-protein diet. *Biochimica et Biophysica Acta* **756**, 182-190.

McFarlane, I.G. & von Holt, C. (1969) Metabolism of amino acids in protein-calorie-deficient rats. *Biochemical Journal* **111**, 557-563.

McNurlan, M.A. & Garlick, P.J. (1989) Influence of nutrient intake on protein turnover. *Diabetes/Metabolism Reviews* **5**, 165-189.

Meguid, M.M., Matthews, D.E., Bier, D.M., Meredith, D.N., Soeldner, J.S. & Young, V.R. (1986) Leucine kinetics at graded leucine intakes in young men. *American Journal of Clinical Nutrition* **43**, 770-780.

Meguid, M.M., Matthews, D.E., Bier, D.M., Meredith, C.N., Soeldner, J.S. & Young, V.R. (1986) Leucine kinetics at graded leucine intakes in young men. *American Journal of Clinical Nutrition* **43**, 770-780.

Meguid, M.M., Matthews, D.E., Bier, D.M., Meredith, C.N. & Young, V.R. (1986) Valine kinetics at graded valine intakes in young men. *American Journal of Clinical Nutrition* **43**, 781-786.

Meikle, A.W. & Klain, G.J. (1972) Effect of fasting and fasting-refeeding on conversion of leucine into CO_2 and lipids in rats. *American Journal of Physiology* **222**, 1246-1250.

Melville, S., McNurlan, M.A., McHardy, K.C., Broom, J., Milne, E., Calder, A.G. & Garlick, P.J. (1989)

The role of degradation in the acute control of protein balance in adult man: Failure of feeding to stimulate protein synthesis as assessed by L-[1-^{13}C]Leucine infusion. *Metabolism* **38**, 248-255.

Meredith, C.N., Wen, Z.-M., Bier, D.M., Matthews, D.E. & Young, V.R. (1986) Lysine kinetics at graded lysine intakes in young men. *American Journal of Clinical Nutrition* **43**, 787-794.

Millward, J. (1990a) Letter to the Editor. Amino acid requirements in adult man. *American Journal of Clinical Nutrition* **51**, 492-494.

Millward, D.J. (1990b) The Arvid Wretlind Lecture 1989. The hormonal control of protein turnover. *Clinical Nutrition* **9**, 115-126.

Millward, D.J. & Rivers, J.P.W. (1988) The nutritional role of indispensable amino acids and the metabolic basis for their requirements. *European Journal of Clinical Nutrition* **42**, 367-393.

Millward, D.J. & Rivers, J.P.W. (1989) The need for indispensable amino acids: the concept of the anabolic drive. *Diabetes/Metabolism Reviews* **5**, 191-211.

Millward, D.J., Nnanyelugo, D.O., James, W.P.T. & Garlick, P.J. (1974) Protein metabolism in skeletal muscle: the effect of feeding and fasting on muscle RNA, free amino acids and plasma insulin concentrations. *British Journal of Nutrition* **32**, 127-142.

Millward, D.J., Price, G.M., Pacy, P.J.H. & Halliday, D. (1990) Maintenance protein requirements: the need for conceptual re-evaluation. *Proceedings of the Nutrition Society* **49**, 473-487.

Millward, D.J., Price, G.M., Pacy, P.J.H. & Halliday, D. (1991a) Whole-body protein and amino acid turnover in man: what can we measure with confidence? *Proceedings of the Nutrition Society* **50**, 197-216.

Millward, D.J., Price, G.M., Pacy, P.J.H., Quevedo, R.M. & Halliday, D. (1991b) The nutritional sensitivity of the diurnal cycling of body protein enables protein deposition to be measured in subjects at nitrogen equilibrium. *Clinical Nutrition* **10**, 239-244.

Mimura, T., Yamada, C. & Swendseid, M.E. (1968) Influence of dietary protein levels and hydrocortisone administration on the branched-chain amino acid transaminase activity in rat tissues. *Journal of Nutrition* **95**, 493-498.

Moore, B.J. & Brasel, J.A. (1984) One cycle of reproduction consisting of pregnancy, lactation or no lactation, and recovery: effects on carcass composition in ad libitum-fed and food-restricted rats. *Journal of Nutrition* **114**, 1548-1559.

Mortimore, G.E. & Pösö, A.R. (1987) Intracellular protein catabolism and its control during nutrient deprivation and supply. *Annual Review of Nutrition* **7**, 539-564.

Motil, K.J., Bier, D.M., Matthews, D.E., Burke, J.F. & Young, V.R. (1981a) Whole body leucine and lysine metabolism studied with [1-^{13}C]Leucine and [α-^{15}N]Lysine: response in healthy young men given excess energy intake. *Metabolism* **30**, 783-791.

Motil, K.J., Matthews, D.E., Bier, D.M., Burke, J.F., Munro, H.N. & Young, V.R. (1981b) Whole-body leucine and lysine metabolism: response to dietary protein intake in young men. *American Journal of Physiology* **240**, E712-E721.

Munro, H.N. (1964a) Historical introduction: The origin and growth of our present concepts of protein metabolism. In: *Mammalian Protein Metabolism*, volume I, pp. 1-29 [H.N. Munro and J.B. Allison, editors]. New York & London: Academic Press.

Munro, H.N. (1964b) General aspects of the regulation of protein metabolism by diet and hormones. In *Mammalian Protein Metabolism*, Volume I, pp. 382-481 [H.N. Munro & J.B. Allison, editors]. New York & London: Academic Press.

Munro, H.N. (1985) Historical perspective on protein requirements: objectives for the future. In: *Nutritional Adaptation in Man*, 155-168 [K. Blaxter and J.C. Waterlow, editors]. London and Paris: John Libbey Publishers.

Nair, K.S., Garrow, J.S., Ford, C., Mahler, R.F. & Halliday, D. (1983) Effect of poor diabetic control and obesity on whole body protein metabolism in man. *Diabetologia* **25**, 400-403.

Nair, K.S., Ford, G.C. & Halliday, D. (1987a) Effect of intravenous insulin treatment on in vivo whole body leucine kinetics and oxygen consumption in insulin-deprived type I diabetic patients. *Metabolism* **36**, 491-495.

Nair, K.S., Halliday, D., Ford, G.C., Heels, S. & Garrow, J.S. (1987b) Failure of carbohydrate to spare leucine oxidation in obese subjects. *International Journal of Obesity* **11**, 537-544.

Nair, K.S., Halliday, D., Matthews, D.E. & Welle, S.L. (1987c) Hyperglucagonemia during insulin deficiency accelerates protein catabolism. *American Journal of Physiology* **253**, E208-E213.

Nair, K.S., Woolf, P.D., Welle, S.L. & Matthews, D.E. (1987d) Leucine, glucose, and energy metabolism after 3 days of fasting in healthy human subjects. *American Journal of Clinical Nutrition* **46**, 557-562.

Nair, K.S., Halliday, D. & Griggs, R.C. (1988) Leucine incorporation into mixed skeletal muscle protein in humans. *American Journal of Physiology* **254**, E208-E213.

Nair, K.S., Welle, S.L., Halliday, D. & Campbell, R.G. (1988) Effect of ß-hydroxybutyrate on whole-body leucine kinetics and fractional mixed skeletal muscle protein synthesis in humans. *Journal of Clinical Investigation* **82**, 198-205.

Naismith, D.J. (1969) The foetus as a parasite. *Proceedings of the Nutrition Society* **28**, 25-31.

Naismith, D.J. & Fears, R.B. (1972) Adaptations in the metabolism of protein during pregnancy in the rat. *Proceedings of the Nutrition Society* **31**, 8A.

Naismith, D.J. & Morgan, B.L.G. (1976) The biphasic nature of protein metabolism during pregnancy in the rat. *British Journal of Nutrition* **36**, 563-566.

Neale, R.J. (1971) Adaptation of amino-acid metabolism in protein-depleted rats. *Nature New Biology* **231**, 117-118.

Neale, R.J. & Waterlow, J.C. (1974) The metabolism of ^{14}C-labelled essential amino acids given by intragastric or intravenous infusion to rats on normal and protein-free diets. *British Journal of Nutrition* **32**, 11-25.

Neale, R.J. & Waterlow, J.C. (1977) Endogenous loss of leucine and methionine in adult male rats. *British Journal of Nutrition* **37**, 259-268.

Nelson, M.M. & Evans, H.M. (1953) Relation of dietary protein levels to reproduction in the rat. *Journal of Nutrition* **51**, 71-84.

Newsholme, E.A., Newsholme, P., Curi, R., Challoner, E. & Ardawi, S. (1988) A role for muscle in the immune system and its importance in surgery, trauma, sepsis and burns. *Nutrition* **4**, 261-268.

Nissen, S. & Haymond, M.W. (1986) Changes in leucine kinetics during meal absorption: effects of dietary leucine availability. *American Journal of Physiology* **250**, E695-E701.

Nissen, S., Van Koevering, M. & Webb, D. (1990) Analysis of ß-hydroxy-ß-methyl butyrate in plasma by gas chromatography and mass spectrometry. *Analytical Biochemistry* **188**, 17-19.

Pacy, P.J., Cheng, K.N., Read, M., Rennie, M.J. & Halliday, D. (1988) Muscle protein synthesis rate - a reappraisal of control values. *Proceedings of the Nutrition Society* **47**, 53A.

Pacy, P.J., Garrow, J.S., Ford, G.C., Merritt, H. & Halliday, D. (1988) Influence of amino acid

administration on whole-body leucine kinetics and resting metabolic rate in postabsorptive normal subjects. *Clinical Science* **75**, 225-231.

Pacy, P.J., Nair, K.S., Ford, C. & Halliday, D. (1989) Failure of insulin infusion to stimulate fractional muscle protein synthesis in type I diabetic patients. *Diabetes* **38**, 618-624.

Pacy, P.J., Cheng, K.N., Ford, G.C. & Halliday, D. (1990) Influence of glucagon on protein and leucine metabolism: a study in fasting man with induced insulin resistance. *British Journal of Surgery* **77**, 791-794.

Pacy, P.J., Thompson, G.N. & Halliday, D. (1991) Measurement of whole-body protein turnover in insulin-dependent (type 1) diabetic patients during insulin withdrawal and infusion: comparison of [^{13}C]leucine and [$^{2}H_5$]phenylalanine methodologies. *Clinical Science* **80**, 345-352.

Paul, G.L. (1989) Dietary protein requirements of physically active individuals. *Sports Medicine* **8**, 154-176.

Paul, H.S. & Adibi, S.A. (1978) Leucine oxidation in diabetes and starvation: effects of ketone bodies on branched-chain amino acid oxidation in vitro. *Metabolism* **27**, 185-200.

Paul, H.S. & Adibi, S.A. (1982) Role of ATP in the regulation of branched-chain α-keto acid dehydrogenase activity in liver and muscle mitochondria of fed, fasted, and diabetic rats. *Journal of Biological Chemistry* **257**, 4875-4881.

Pelletier, V., Marks, L., Wagner, D.A., Hoerr, R.A. & Young, V.R. (1991) Branched-chain amino acid interactions with reference to amino acid requirements in adult men: leucine metabolism at different valine and isoleucine intakes. *American Journal of Clinical Nutrition* **54**, 402-407.

Picou, D. & Taylor-Roberts, T. (1969) The measurement of total protein synthesis and catabolism and nitrogen turnover in infants in different nutritional states and receiving different amounts of dietary protein. *Clinical Science* **36**, 283-296.

Poo, L.J., Lew, W., Lee, D.D. & Addis, T. (1940) Protein anabolism in the organs and tissues of pregnant rats at different levels of protein consumption. *Journal of Nutrition* **19**, 505-515.

Pösö, A.R. & Mortimore, G.E. (1985) Amino acid control of hepatic protein degradation: novel requirement for alanine. In: *Intracellular Protein Catabolism*, pp 427-436 [E.A. Khairallah, J.S. Bond and J.W.C. Bird, editors]. New York: Alan R. Liss.

Preedy, V.R. & Garlick, P.J. (1986) The response of muscle protein synthesis to nutrient intake in postabsorptive rats: the role of insulin and amino acids. *Bioscience Reports* **6**, 177-183.

Price, G., Millward, D.J., Pacy, P.J.H. & Halliday, D. (1990) Whole-body protein homeostasis and protein requirements in man: validation studies of a new metabolic model. *Proceedings of the Nutrition Society* **49**, 194A.

Rennie, M.J., Halliday, D., Davies, C.T.M., Edwards, R.H.T., Krywawych, S., Millward, D.J. & Matthews, D.E. (1981) Exercise induced increase in leucine oxidation in man and the effect of glucose. In: *Metabolism and Clinical Implications of Branched Chain Amino and Ketoacids*, pp. 361-366 [M. Walser & J.R. Williamson, editors]. New York: Elsevier North-Holland.

Rennie, M.J., Edwards, R.H.T., Halliday, D., Matthews, D.E., Wolman, S.L. & Millward, D.J. (1982) Muscle protein synthesis measured by stable isotope techniques in man: the effects of feeding and fasting. *Clinical Science* **63**, 519-523.

San Pietro, A. & Rittenberg, D. (1953) A study of the rate of protein synthesis in humans. II. Measurement of the metabolic pool and the rate of protein synthesis. *Journal of Biological Chemistry* **201**, 457-473.

Scheurink, A.J.W., Steffens, A.B., Dreteler, G., Benthem, L. & Bruntink, R. (1989) Experience affects exercise-induced changes in catecholamines, glucose and FFA. *American Journal of Physiology* **256**, R169-R173.

Schoenheimer, R., Ratner, S. & Rittenberg, D. (1939) Studies in protein metabolism. VII. The metabolism of tyrosine. *Journal of Biological Chemistry* **127**, 333-344.

Schreurs, V.V.A.M., Krawielitzki, K., Boekholt, H.A., Koopmanschap, R.E. & Weijs, P.J.M. (1991) Recovery of metabolic end products from 14C & 15N leucine: A comparison at different levels of protein intake. In: *Proceedings of the 6th EAAP International Symposium on Protein Metabolism and Nutrition*, pp. 139-141.

Schreurs, V.V.A.M., Boekholt, H.A., Koopmanschap, R.E. & Weijs, P.J.M. (1992) The metabolic utilization of amino acids: potentials of $^{14}CO_2$ breath test measurements. *British Journal of Nutrition* **67**, 207-214.

Schwenk, W.F., Beaufrere, B. & Haymond, M.W. (1985) Use of reciprocal pool specific activities to model leucine metabolism in humans. *American Journal of Physiology* **249**, E646-E650.

Schwenk, W.F., Tsalikian, E., Beaufrere, B. & Haymond, M.W. (1985) Recycling of an amino acid label with prolonged isotope infusion: implications for kinetic studies. *American Journal of Physiology* **248**, E482-E487.

Shangraw, R.E., Stuart, C.A., Prince, M.J., Peters, E.J. & Wolfe, R.R. (1988) Insulin responsiveness of protein metabolism in vivo following bedrest in humans. *American Journal of Physiology* **255**, E548-E558.

Simon, O. & Bergner, H. (1983) Veränderungen der Proteinsynthese in Leber und Dünndarm von Ratten nach Futteraufnahme. *Biomedica et Biochimica Acta* **42**, 1299-1308.

Simon, O. (1989) Metabolism of proteins and amino acids. In: *Protein Metabolism in Farm Animals. Evaluation, Digestion, Absorption, and Metabolism*, pp 273-366 [H.-D. Bock, B.O. Eggum, A.G. Low, O. Simon and T. Zebrowska, editors]. Oxford & Berlin: Oxford Science Publications & VEB Deutscher Landwirtschaftsverlag.

Sketcher, R.D., Fern, E.B. & James, W.P.T. (1974) The adaptation in muscle oxidation of leucine to dietary protein and energy intake. *British Journal of Nutrition* **31**, 333-342.

Sneible, P.A., Airhart, J. & Low, R.B. (1981) Differential compartmentation of leucine for oxidation and for protein synthesis in cultured skeletal muscle. *Journal of Biological Science* **256**, 4888-4894.

Snell, K. (1980) Muscle alanine synthesis and hepatic gluconeogenesis. *Biochemical Society Transactions* **8**, 205-213.

Spiteri, N.J., Alingh Prins, A.B., Keyser, J. & Strubbe, J.H. (1982) Circadian pacemaker control of feeding in the rat, at dawn. *Physiology & Behavior* **29**, 1141-1145.

Sprinson, D.B. & Rittenberg, D. (1949) The rate of interaction of the amino acids of the diet with the tissue proteins. *Journal of Biological Chemistry* **180**, 715-726.

SPSS Inc. (1988). *SPSS/PC+ Base manual.* Chicago, Illinois: SPSS Inc.

Steffens, A.B. (1969) A method for frequent sampling of blood and continuous infusion of fluids in the rat without disturbing the animal. *Physiology & Behavior* **4**, 833-836.

Stryer, L. (1988) *Biochemistry*, 3rd ed.. New York: Freeman and Co.

Tarnopolsky, M.A., Atkinson, S.A., MacDougall, J.D., Senor, B.B., Lemon, P.W.R. & Schwarcz, H. (1991) Whole body leucine metabolism during and after resistance exercise in fed humans.

Medicine and Science in Sports and Exercise **23**, 326-333.

Thompson, G.N., Pacy, P.J., Ford, G.C., Merritt, H. & Halliday, D. (1988) Relationship between plasma isotope enrichments of leucine and α-ketoisocaproic acid during continuous infusion of labelled leucine. *European Journal of Clinical Investigation* **18**, 639-643.

Thompson, G.N., Pacy, P.J., Merritt, H., Ford, G.C., Read, M.A., Cheng, K.N. & Halliday, D. (1989) Rapid measurement of whole body and forearm protein turnover using a [$^2H_5$]phenylalanine model. *American Journal of Physiology* **256**, E631-E639.

Todd, K.S, Butterfield, G.E. & Calloway, D.H. (1984) Nitrogen balance in men with adequate and deficient energy intake at three levels of work. *Journal of Nutrition* **114**, 2107-2118.

Tsalikian, E., Howard, C., Gerich, J.E. & Haymond, M.W. (1984) Increased leucine flux in short-term fasted human subjects: evidence for increased proteolysis. *American Journal of Physiology* **247**, E323-E327.

Vazquez, J.A., Harbhajan, S.P. & Adibi, S.A. (1986) Relation between plasma and tissue parameters of leucine metabolism in fed and starved rats. *American Journal of Physiology* **250**, E615-E621.

Verboeket-van de Venne, W.P.H.G. & Westerterp, K.R. (1991) Influence of the feeding frequency on nutrient utilization in man. Consequences for energy metabolism. *European Journal of Clinical Nutrition* **45**, 161-169.

Viru, A. (1987) Mobilisation of structural proteins during exercise. *Sports Medicine* **4**, 95-128.

Wahren, J., Felig, P. & Hagenfeldt, L. (1976) Effect of protein ingestion on splanchnic and leg metabolism in normal man and in patients with diabetes mellitus. *Journal of Clinical Investigation* **57**, 987-999.

Waterlow, J.C. & Stephen, J.M.L. (1967) The measurement of total lysine turnover in the rat by intravenous infusion of L-[U-^{14}C]lysine. *Clinical Science* **33**, 489-506.

Waterlow, J.C. (1967) Lysine turnover in man measured by intravenous infusion of L-[U-^{14}C]lysine. *Clinical Science* **33**, 507-515.

Waterlow, J.C. (1968) Observations on the mechanism of adaptation to low protein intakes. *Lancet* **ii**, 1091-1097.

Waterlow, J.C., Garlick, P.J. & Millward, D.J. (1978) *Protein Turnover in Mammalian Tissues and in the Whole Body*, pp. 319/529-594. Amsterdam, New York & Oxford: North-Holland Publishing Company.

Wernerman, J., von der Decken, A. & Vinnars, E. (1985) The diurnal pattern of protein synthesis in human skeletal muscle. *Clinical Nutrition* **4**, 203-205.

White, T.P. & Brooks, G.A. (1981) [U-^{14}C]glucose, -alanine, and -leucine oxidation in rats at rest and two intensities of running. *American Journal of Physiology* **240**, E155-E165.

Wolfe, R.R. (1984) *Tracers in Metabolic Research. Radioisotope and Stable Isotope/Mass Spectrometry Methods*. Laboratory and Research Methods in Biology and Medicine, Volume 9. New York: Alan R. Liss.

Wolfe, R.R. & Jahoor, F. (1990) Recovery of labeled CO_2 during the infusion of C-1- vs C-2-labeled acetate: implications for tracer studies of substrate oxidation. *American Journal of Clinical Nutrition* **51**, 248-252.

Wolfe, R.R., Goodenough, R.D., Wolfe, M.H., Royle, G.T. & Nadel, E.R. (1982) Isotopic analysis of leucine and urea metabolism in exercising humans. *Journal of Applied Physiology* **52**, 458-466.

Wolfe, R.R., Wolfe, M.H., Nadel, E.R. & Shaw, J.H.F. (1984) Isotopic determination of amino acid-

urea interactions in exercise in humans. *Journal of Applied Physiology* **56**, 221-229.

Wolfram, G., Kirchgeßner, M., Müller, H.L. & Hollomey, S. (1987) Thermogenese des Menschen bei unterschiedlicher Mahlzeitenhäufigkeit. *Annals of Nutrition and Metabolism* **31**, 88-97.

Yagi, M. & Walser, M. (1990) Estimation of whole body protein synthesis from oxidation of infused [1-^{14}C]leucine. *American Journal of Physiology* **258**, E151-E157.

Yoshida, S., Lanza-Jacoby, S. & Stein, T.P. (1991) Leucine and glutamine metabolism in septic rats. *Biochemical Journal* **276**, 405-409.

Young, V.R. (1986). Nutritional balance studies: indicators of human requirements or of adaptive mechanisms? *Journal of Nutrition* 116: 700-703.

Young, V.R. (1987) 1987 McCollum Award Lecture. Kinetics of human amino acid metabolism: nutritional implications and some lessons. *American Journal of Clinical Nutrition* **46**, 709-725.

Young, V.R. & Bier, D.M. (1987) A kinetic approach to the determination of human amino acid requirements. *Nutrition Reviews* **45**, 289-298.

Young, V.R., Gucalp, C., Rand, W.M., Matthews, D.E. & Bier, D.M. (1987) Leucine kinetics during three weeks at submaintenance-to-maintenance intakes of leucine in men: adaptation and accomodation. *Human Nutrition: Clinical Nutrition* 41C, 1-18.

Young, V.R., Bier, D.M. & Pellett, P.L. (1989) A theoretical basis for increasing current estimates of the amino acid requirements in adult man, with experimental support. *American Journal of Clinical Nutrition* **50**, 80-92.

Young, V.R., Bier, D. & Pellett, P. (1990) Reply to J. Millward. *American Journal of Clinical Nutrition* **51**, 494-496.

Young, V.R. & Marchini, J.S. (1990) Mechanisms and nutritional significance to altered intakes of protein and amino acids, with reference to nutritional adaptation in humans. *American Journal of Clinical Nutrition* **51**, 270-289.

Young, V.R. (1991) Nutrient interactions with reference to amino acid and protein metabolism in non-ruminants; particular emphasis on protein-energy relations in man. *Zeitschrift für Ernährungswissenschaft* **30**, 239-267.

Young, V.R. & Pellett, P.L. (1991) Protein evaluation, amino acid scoring and the food and drug administration's proposed food labeling regulations. *Journal of Nutrition* **121**, 145-150.

Yu, Y.M., Wagner, D.A., Tredget, E.E., Walaszewski, J.A., Burke, J.F. & Young, V.R. (1990) Quantitative role of splanchnic region in leucine metabolism: L-[1-^{13}C,^{15}N] leucine and substrate balance studies. *American Journal of Physiology* **259**, E36-E51.

Zhao, X., Wen, Z.-M., Meredith, C.N., Matthews, D.E., Bier, D.M. & Young, V.R. (1986) Threonine kinetics at graded threonine intakes in young men. *American Journal of Clinical Nutrition* **43**, 795-802.

SUMMARY

This thesis describes effects of dietary protein supply and physiological condition of the animal on metabolic amino acid utilisation. The aim of this study was to identify the causes and objectives of amino acid losses and therefore the physiological or metabolic basis for their nutritional requirements. Since current methodology did not provide the means to study the physiological non-steady state, we employed a simple new technique in order to obtain data on the efficiency of metabolic amino acid utilisation. The technique essentially consisted of a constant infusion of ^{14}C-leucine and simultaneous collection of expired $^{14}CO_2$. The expired amount of radioactivity expressed as percentage of the infused dose reflected the percental oxidation of the labelled leucine pool. Perturbation of the isotopic steady state is studied by ingestion of a meal or the commencement of exercise; the physiological non-steady state.
Since the results are also influenced by changes in the specific activity of the free leucine pool and the ratio of expiration and production of $^{14}CO_2$ (recovery), the data should be interpreted with care. Basically any tracer study faces these problems. At present we emphasize the importance of the nutritional and physiological causes of amino acid losses, and consider the limitations of the technique acceptable and without effect on the interpretation of the results.

The first study showed that the percental oxidation of leucine increased acutely upon ingestion of a meal. While the postabsorptive percental oxidation was determined by the previous dietary protein level, the postprandial percental oxidation was determined by the protein level of the meal. This indicated that the percental oxidation of leucine was very sensitive to changes in the nutritional status.
The high protein diet resulted in a high percental oxidation after an overnight fast. The hypothesis that the high protein diet resulted in a high postprandial protein gain and thus (in mature animals) in a high postabsorptive protein loss, is in line with this observation.
Growing animals showed a significant lower percental oxidation in the postabsorptive state, compared to mature animals. The response of leucine oxidation to feeding was essentially the same for growing and mature animals. However, in the early postprandial phase (2-4 h after onset of the meal) the growing animals showed a significant lower percental oxidation. This is in line with the ability of growing animals to increase protein synthesis upon feeding.
The difference between the percental decarboxylation and percental total oxidation of leucine increased after ingestion of a meal. This difference increased more with meals

with a higher protein level and with the mature animals. This indicated a relationship between this difference and the level of amino acid oversupply relative to the requirement of the animal.

The second study showed that the percental decarboxylation of leucine increased with prolonged (more than overnight) fasting, especially for the low protein group. A protein free meal reversed the increased level of decarboxylation to the lower overnight fasted level. However, the percental total oxidation was not significantly altered. This indicated that leucine does play a role during energy shortage, but not by direct oxidation of the carbon skeleton. Leucine nitrogen can be used to generate substrates for the gut (glutamine) and liver gluconeogenesis (alanine), while leucine carbon can be used for liver ketogenesis. It may also be that the enzyme activity for decarboxylation of leucine is increased because isoleucine and valine supply substrates for gluconeogenesis.
With constant intravenous tracer infusion, prolonged fasting increased the percental leucine oxidation only at 6 h after the meal. When tracer was given with the meal the percental leucine oxidation was much higher when a longer fasting period preceded the meal. This indicated that primarily the oxidation of dietary leucine was increased. It is striking that the effect of prolonged fasting persists into the postprandial state.

In the third study the leucine tracer was ingested with the meal. In the early postprandial phase (1-2 h after the meal) the percental oxidation of leucine was lower for growing compared to mature animals. In this period the percental oxidation was not different between diets. However, after this period the percental oxidation decreased for growing animals on a low protein diet, which resulted in a significant lower cumulative percental oxidation. These observations were all in line with the first study.
Both the percental decarboxylation and percental total oxidation of leucine were increased after a meal enriched with synthetic leucine. The difference between decarboxylation and total oxidation was not increased, except for the first hour. This indicated that the difference did not reflect a specific leucine oversupply, but a general amino acid oversupply as suggested in the first study. After ingestion of a very high protein meal, which contained a similar amount of leucine, the percental decarboxylation was similar but delayed. This indicated a difference in metabolic appearance between the synthetic and the protein-bound leucine.

The fourth study showed that pregnancy decreased the percental oxidation of leucine

in the postabsorptive state. After ingestion of a low protein meal the percental oxidation was lower at day 18 of pregnancy compared to before pregnancy, but did not reach statistical significance. These observations indicated that pregnancy in mature animals caused similar changes in leucine metabolism as were observed for growth in the first study.
This experiment was performed with animals conditioned on the high protein diet, since the low protein diet did not meet pregnancy requirements. Only one pregnancy was successful. Four young females were born and fed the low protein diet. When these females changed to the high protein diet at 6 weeks, the growth rate improved rapidly to normal.
Both aspects show the remarkable capacity for adaptation to the dietary protein level.

The fifth study showed that the percental leucine oxidation in the postabsorptive state was higher for animals conditioned to a meal frequency of 5 small meals compared to 2 large meals. This indicated that more frequent small meals decreased leucine reutilisation in the postabsorptive state. And this suggested a larger postabsorptive protein loss and a larger postprandial protein gain, in other words an increased protein cycling.
During the 10 h feeding period the only difference between the two meal frequencies was the fast decrease in the percental oxidation several hours after both large meals. The percental oxidation in the subsequent postabsorptive period remained much higher for the frequent small meals.
Furthermore the expiration of $^{14}CO_2$ had become twice the initial level which indicated a large recycling of tracer. When tracer infusion during the initial postabsorptive period was prolonged from 2 to 10 h, the expiration of $^{14}CO_2$ in this period increased significantly. During the feeding period however there was no difference in expired $^{14}CO_2$. This indicated that tracer recycling was not a problem during feeding and suggested a decreased protein breakdown during feeding. The expiration of $^{14}CO_2$ increased immediately after the feeding period with large meals. This indicated that protein breakdown increased again after the large meals. It was therefore concluded that large meals increased the variation in protein breakdown.

In the sixth study the percental leucine oxidation increased acutely after 60 min swimming exercise and for several hours thereafter. Since the plasma leucine pool was also increased, this indicated that leucine oxidation increased after exercise. This suggested that exercise increased the postabsorptive protein loss. However, 10 min swimming resulted in the same response, which suggested that 'metabolic stress' was

more important in this experiment than endurance exercise.
Training increased the response to exercise for the low protein group and decreased it for the high protein group. Training did not increase feed intake in either group. The response to a high protein meal was also largely unaltered by training. However, training decreased the percental oxidation at 6 h after the meal. This indicated that training increased the postprandial protein gain. It was suggested that training would need to have an even larger effect in the low protein group.

In general the percental oxidation of leucine varied widely with different physiological conditions and nutritional manipulations. This indicated a large diurnal variation in amino acid losses, which have to be accounted for in estimations of nutritional requirements.
The postprandial percental oxidation was determined by the protein content of the meal. This indicated that estimations of nutritional requirements could not be based on postprandial measurements, since they primarily reflected the level of dietary protein intake and to a minor extent the physiological condition.

SAMENVATTING

Dit proefschrift beschrijft de invloed van voedingseiwit en de fysiologische conditie van het dier op de metabole benutting van aminozuren. Het doel van deze studie was het identificeren van oorzaken van verliezen van essentiele aminozuren, het doel daarvan en daarmee de fysiologische of metabole oorzaak voor de behoefte hieraan. Omdat er geen methode beschikbaar is om de fysiologische non-steady state te bestuderen, hebben we een eenvoudige nieuwe methode gebruikt waarmee data over de efficientie van de metabole benutting van aminozuren kunnen worden verkregen. De methode bestaat in essentie uit een constant infuus van ^{14}C-leucine en het tegelijkertijd opvangen van de uitgeademde $^{14}CO_2$. De uitgeademde radioactiviteit wordt uitgedrukt als percentage van de geinfuseerde dosis, het percentage oxidatie van leucine. Als een isotopen steady-state is bereikt, dan kan deze verstoord worden door het consumeren van een maaltijd of door lichamelijke inspanning; de fysiologische steady-state.
Omdat de resultaten sterk afhankelijk zijn van veranderingen in de labelling van de vrije aminozuur pool en van de correlatie tussen produktie en uitademing van $^{14}CO_2$, moet bij de interpretatie van de resultaten enige voorzichtigheid in acht worden genomen. Deze problemen zijn inherent aan isotopenstudies. Momenteel benadrukken we het belang van de door de voeding en de fysiologische conditie veroorzaakte verliezen aan aminozuren, en daarom beschouwen we de beperkingen van de methode als acceptabel en zonder invloed op de interpretatie van de resultaten.

Het eerste onderzoek gaf aan dat het percentage oxidatie van leucine acuut toenam tijdens een maaltijd. Terwijl het postabsorptieve percentage oxidatie werd bepaald door het eiwitgehalte van de voorgaande voeding, werd het postprandiale percentage oxidatie bepaald door het eiwitgehalte van de maaltijd. Dit gaf aan dat het percentage oxidatie van leucine zeer gevoelig was voor veranderingen in de voedingstoestand.
Het eiwitrijke voer resulteerde in een hoog oxidatiepercentage na een nacht vasten. De hypothese dat het eiwitrijke voer resulteerde in een hoge postprandiale eiwitaanzet en dus (in volwassen dieren) in een hoog postabsorptief eiwitverlies, is in overeenstemming met deze waarneming.
Bij groeiende dieren werd een significant lager oxidatiepercentage gevonden in de postabsorptieve toestand, vergeleken met volwassen dieren. Het voedingseffect op de leucine oxidatie was in essentie gelijk bij groeiende en volwassen dieren. Hoewel, in de vroege postprandiale fase (2-4 h na het begin van de maaltijd) vertoonden de

groeiende dieren een significant lager oxidatiepercentage. Dit is in overeenstemming met het vermogen van groeiende dieren om de eiwitsynthese te verhogen tijdens de absorptie.
Het verschil tussen het percentage decarboxylatie en het percentage volledige oxidatie van leucine neemt toe na een maaltijd. Dit verschil nam meer toe bij maaltijden met een hoger eiwitgehalte en bij volwassen dieren. Dit gaf een relatie aan tussen dit verschil en de mate van aminozuuraanbod relatief ten opzichte van de behoefte van het dier.

Het tweede onderzoek gaf aan dat het percentage decarboxylatie van leucine toenam als de periode van vasten langer was dan één nacht, vooral bij de eiwitarme groep. Een eiwitvrije maaltijd liet dit oxidatieniveau weer dalen tot het niveau na één nacht vasten. Maar het percentage volledige oxidatie veranderde niet significant. Dit gaf aan dat leucine een rol speelt bij een tekort aan energie, maar niet door verbranding van het koolstof-skelet. Leucine stikstof kan worden gebruikt voor de synthese van substraat voor de darm (glutamine) en lever gluconeogenese (alanine), terwijl leucine koolstof kan worden gebruikt voor lever ketogenese. Het kan ook zijn dat de enzymactiviteit voor decarboxylatie van leucine toeneemt omdat isoleucine en valine substraat voor gluconeogenese leveren.
Bij een constant intraveneus tracer infuus, resulteerde langer vasten in een hoger percentage oxidatie van leucine 6 h na de maaltijd. Als de tracer met de maaltijd werd gegeven, was het oxidatiepercentage veel hoger bij een langere periode van vasten vooraf aan de maaltijd. Dit gaf aan dat vooral de oxidatie van leucine uit de voeding toenam. Het was opvallend dat het effect van langer vasten voortduurde tot in de postprandiale toestand.

In het derde onderzoek werd de leucine tracer met een maaltijd gegeven. In de vroege postprandiale fase (1-2 h na de maaltijd) was het percentage oxidatie van leucine lager voor groeiende vergeleken met volwassen dieren. In deze periode was het oxidatiepercentage niet verschillend tussen de voersoorten. Hoewel, na deze periode nam het oxidatiepercentage af voor groeiende dieren op een eiwitarm voer en dat resulteerde in een significant lager cumulatief oxidatiepercentage. Deze waarnemingen waren in overeenstemming met het eerste onderzoek.
Zowel het percentage decarboxylatie en het percentage volledige oxidatie van leucine namen toe na eem maaltijd verrijkt met synthetisch leucine. Het verschil tussen decarboxylatie en volledige oxidatie nam niet toe, behalve in het eerste uur. Dit gaf aan dat het verschil niet specifiek het overschot aan leucine weergaf, maar een

algemeen overschot aan aminozuren zoals door het eerste onderzoek werd gesuggereerd. Na een zeer eiwitrijke maaltijd, met een vergelijkbaar leucinegehalte, was het percentage decarboxylatie vergelijkbaar maar vertraagd. Dit gaf een verschil aan in metabole beschikbaarheid tussen het synthetische en eiwitgebonden leucine.

Het vierde onderzoek gaf aan dat zwangerschap (de dracht) het percentage oxidatie van leucine verlaagde in de postabsorptieve toestand. Na een eiwitarme maaltijd was het oxidatiepercentage lager op dag 18 van de dracht vergeleken bij de periode voor de dracht, maar dit was statistisch niet significant. Deze waarnemingen gaven aan dat de dracht bij volwassen dieren vergelijkbare veranderingen veroorzaakt in het leucine metabolisme als groei in het eerste onderzoek.
Dit experiment werd uitgevoerd met dieren geconditioneerd op een eiwitrijk voer, omdat het eiwitarme voer niet toereikend was voor de dracht. Slechts één dracht was succesvol. Vier jonge vrouwtjes werden geboren, die het eiwitarme voer kregen. Toen deze vrouwtjes na 6 weken het eiwitrijke voer werd verstrekt, werd de groeisnelheid snel normaal.
Beide aspekten geven aan dat er een enorm vermogen is tot aanpassing aan het eiwitgehalte van de voeding.

Het vijfde onderzoek gaf aan dat het percentage oxidatie van leucine in de postabsorptieve toestand hoger was voor een maaltijdfrequentie van 5 kleine maaltijden vergeleken bij 2 grote maaltijden. Dit gaf aan dat meer kleine maaltijden de herbenutting van leucine in de postabsorptieve toestand verminderde. En dit suggereerde een groter postabsorptief verlies en een grotere postprandiale eiwitaanzet, met andere woorden een grotere 'eiwitcyclus'.
Tijdens de 10 urige voedingsperiode was het enige verschil tussen de twee maaltijd frequenties dat het oxidatiepercentage enkele uren na beide grote maaltijden afnam. Het oxidatiepercentage in de volgende postabsorptieve periode bleef veel hoger na meer kleine maaltijden.
Verder verdubbelde de hoeveelheid uitgeademde $^{14}CO_2$ en dat gaf een grote mate van tracer recycling aan. Als het tracer infuus tijdens de eerste postabsorptieve periode werd verlengd van 2 naar 10 h, nam de uitgeademde $^{14}CO_2$ significant toe in deze periode. Tijdens de voedingsperiode was er geen verschil in uitgeademde $^{14}CO_2$. Dit gaf aan dat tracer recycling geen probleem was tijdens de voeding. De hoeveelheid uitgeademde $^{14}CO_2$ nam direkt na de voedingsperiode toe bij de grote maaltijden. Daarom werd geconcludeerd dat bij de grote maaltijden de variatie in eiwitafbraak toenam.

In het zesde onderzoek nam het percentage oxidatie van leucine acuut toe na 60 min zweminspanning en gedurende enkele uren daarna. Omdat de plasma leucine pool ook toenam, gaf dit aan dat oxidatie van leucine toenam na lichamelijke inspanning. Dit suggereerde dat lichamelijke inspanning het postabsorptieve eiwitverlies verhoogd. Echter, een zweminspanning van 10 min resulteerde in dezelfde response en dat suggereerde dat 'metabole stress' belangrijker was in dit experiment dan duurinspanning.
Training verhoogde de response op lichamelijke inspanning bij de eiwitarme groep en verlaagde dit bij de eiwitrijke groep. De voeropname werd in geen van beide groepen verhoogd door training. De response op een eiwitrijke maaltijd werd nauwelijks beïnvloed door training. Hoewel, training verlaagde het oxidatiepercentage 6 h na de maaltijd. Dit gaf aan dat training de postprandiale eiwitaanzet verhoogde. Gesuggereerd werd dat dit trainingseffect zelfs sterker zou moeten zijn in de eiwitarme groep.

In het algemeen varieerde het percentage oxidatie van leucine sterk met de verschillende fysiologische toestanden en veranderingen in de voeding. Dit gaf aan dat aminozuurverliezen sterk variëren over de dag en daarmee moet rekening worden gehouden in de schatting van de voedingsbehoefte.
Het postprandiale oxidatiepercentage werd bepaald door het eiwitgehalte van de maaltijd. Dit gaf aan dat schattingen van de voedingsbehoefte niet kunnen worden gebaseerd op postprandiale metingen, omdat ze voornamelijk de eiwitinname met de voeding weergaven en in geringe mate de fysiologische toestand.

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LIST OF PUBLICATIONS

Research Papers

Maas, I.A.M., Schreurs, V.V.A.M., Weijs, P.J.M., Frings, M., van Acker, B.A.C., Boekholt, H.A. & Koopmanschap, R.E. (1989) Combined effects of dietary restrictions and physical activity on amino acid utilization in rats. *Netherlands Journal of Agricultural Sciences* **37**, 263-268.

Weststrate, J.A., Weys, P.J.M., Poortvliet, E.J., Deurenberg, P. & Hautvast, J.G.A.J. (1989) Diurnal variation in postabsorptive resting metabolic rate and diet-induced thermogenesis. *American Journal of Clinical Nutrition* **50**, 908-914.

Weststrate, J.A., Weys, P., Poortvliet, E., Deurenberg, P. & Hautvast, J.G.A.J. (1990) Lack of a systemic sustained effect of prolonged exercise bouts on resting metabolic rate in fasting subjects. *European Journal of Clinical Nutrition* **44**, 91-97.

Schreurs, V.V.A.M., Boekholt, H.A., Koopmanschap, R.E. & Weijs, P.J.M. (1992) The metabolic utilization of amino acids. Potentials of $^{14}CO_2$ breath test measurements. *British Journal of Nutrition* **67**, 207-214.

Weijs, P.J.M., Schreurs, V.V.A.M., Koopmanschap, R.E., Grooten, H.N.A., Schoonman, A.T. & Boekholt, H.A. (1993) Effects of acute and chronic protein restriction on metabolic leucine utilisation in growing and mature rats. *British Journal of Nutrition* (in press).

Weijs, P.J.M. & Schreurs, V.V.A.M. Effect of fasting and feeding on metabolic leucine utilisation. Submitted.

Weijs, P.J.M., Schreurs, V.V.A.M., van der Kamp, M.D.O. & Koopmanschap, R.E. Effects of protein restriction and synthetic leucine on postprandial metabolic leucine utilisation in growing and mature rats. Submitted.

Weijs, P.J.M., Schreurs, V.V.A.M. & Grooten, H.N.A. Effect of pregnancy on metabolic leucine utilisation. Submitted.

Weijs, P.J.M., Schreurs, V.V.A.M. & Maas, M.I.M. Effect of meal frequency and tracer infusion time on metabolic leucine utilisation. Submitted.

Weijs, P.J.M., Schreurs, V.V.A.M., Helmes, M.H.B., Bujko, J., Koopmanschap, R.E., Boekholt, H.A. & Keller, J. Effects of protein restriction on meal- and exercise-induced changes in metabolic leucine utilisation. Submitted.

Short Papers

Weijs, P.J.M., Schreurs, V.V.A.M., Boekholt, H.A. & Koopmanschap, R.E. (1989) Verschil in kinetiek tussen metabole benutting van synthetische en eiwitgebonden aminozuren. *Proceedings 14de Studiedag Nederlandstalige Voedingsonderzoekers*, 34-35.

Weijs, P.J.M., Schreurs, V.V.A.M., Boekholt, H.A. & Koopmanschap, R.E. (1990) Veranderingen in de benutting van aminozuren tijdens en na een maaltijd. *Proceedings 15de Studiedag Nederlandstalige Voedingsonderzoekers*, Utrecht, 1990:35-36.

Schreurs, V.V.A.M., Krawielitzki, K., Boekholt, H.A., Koopmanschap, R.E. & Weijs, P.J.M. (1991) Recovery of metabolic end products from [^{14}C] & [^{15}N] leucine. A comparison at different levels of protein intake. *Proceedings 6th EAAP International Symposium on Protein Metabolism and Nutrition*, 139-141.

Weijs, P.J.M., Schreurs, V.V.A.M., Grooten, H.N.A., Koopmanschap, R.E. & Boekholt H.A. (1991) Meal-induced changes in leucine oxidation in growing rats as influenced by protein level in diet and test meal. *Proceedings 6th EAAP International Symposium on Protein Metabolism and Nutrition*, 142-144.

Schreurs, V.V.A.M., Krawielitzki, K., Boekholt, H.A., Koopmanschap, R.E. & Weijs, P.J.M. (1991) "In vivo" stofwisselingsonderzoek met gemerkte aminozuren; een pilot studie met [^{14}C] en [^{15}N] leucine. *Proceedings 16de Studiedag Nederlandstalige Voedingsonderzoekers*, 32-33.

Weijs, P.J.M., Schreurs, V.V.A.M., Koopmanschap, R.E., Grooten, H.N.A., Schoonman, A.T. & Boekholt, H.A. (1991) Maaltijd geinduceerde veranderingen in metabole benutting van leucine bij jonge groeiende en volwassen ratten. *Proceedings 16de Studiedag Nederlandstalige Voedingsonderzoekers*, 34-35.

Weijs, P.J.M., Schreurs, V.V.A.M., van der Kamp, M.D.O., Koopmanschap, R.E. & Boekholt, H.A. (1992) De invloed van de fysiologische toestand van het dier op de benutting van aminozuren na een maaltijd. *Proceedings 17de Studiedag Nederlandstalige Voedingsonderzoekers*, 27-28.

Abstracts

Schreurs, V.V.A.M., Boekholt, H.A., Koopmanschap, R.E., Jansen, J., Poortvliet, E.J. & Weys, P.J.M. (1987) The effect of swim training on amino acid utilization at different levels of dietary protein. *Pflügers Archiv European Journal of Physiology*, S32.

Weijs, P.J.M., Schreurs, V.V.A.M., van der Kamp, M.D.O., Boekholt, H.A. & Koopmanschap, R.E. (1989) Postprandial amino acid utilization depends on physiological conditions. *Pflügers Archiv European Journal of Physiology* S200.

Weijs, P.J.M., Schreurs, V.V.A.M., Koopmanschap, R.E. & Boekholt, H.A. (1990) Aminozuuroxydatie voor, tijdens en na een maaltijd. *Netherlands Journal of Nutrition* **51**, 19-20.

Weijs, P.J.M., Schreurs, V.V.A.M., Koopmanschap, R.E., Slingerland, E.W.A. & Boekholt, H.A. (1990) Exercise-induced changes in amino acid oxidation in relation to dietary protein level. *Proceedings 24th FIMS World Congress of Sports Medicine*, 163.

Schreurs, V.V.A.M., Boekholt, H.A., Blaak, E., Weijs, P.J.M. & Koopmanschap, R.E. (1991) Post-exercise leucine catabolism in relation to the nutritional state. *Medicine and Science in Sports and Exercise* **23**, S79.

Weijs, P.J.M., Schreurs, V.V.A.M. & Maas, M.I.M. (1991) Effect of meal frequency and tracer infusion time on metabolic utilization of leucine. *Clinical Nutrition* **10**, 28 (Special Supplement 2:P5).

Weijs, P.J.M. & Schreurs, V.V.A.M. (1992) Discrepancy between the effect of intravenous and oral administration of tracer on leucine oxidation measurements. *Phlügers Archiv European Journal of Physiology* **421**: R43.

Weijs, P.J.M., Schreurs, V.V.A.M., Helmes, M.H.B., Bujko, J., Koopmanschap, R.E., Boekholt, H.A. & Keller, J. (1992) Effect of fasting and feeding on metabolic utilisation of leucine. *Clinical Nutrition* **11**, 51 (Special Supplement:P21).

Schreurs, V.V.A.M., van den Heuvel, E.G.H.M., Krawielitzki, K., Bujko, J., Weijs, P.J.M., Koopmanschap, R.E. & Keller, J. (1993) Amino acid catabolism at rest in relation to dietary protein level and treadmill training. *Medicine and Science in Sports and Exercise* (Submitted).

CURRICULUM VITAE

Peter Johan Marie Weijs werd op 11 februari 1964 geboren in Horst. In 1982 behaalde hij het diploma Atheneum aan het Peelland College in Deurne en begon zijn studie aan de toenmalige Landbouwhogeschool in Wageningen. In januari 1988 sloot hij zijn studie Voeding van de Mens af, met als hoofdvakken Fysiologie van Mens en Dier en Voeding van de Mens.

Vanaf april 1988 was hij werkzaam als Assistent in Opleiding (AIO) bij de Vakgroep Fysiologie van Mens en Dier aan de Landbouwuniversiteit in Wageningen. Hij werkte van april tot augustus 1988 op de Metabolism Unit van het Shriners Burns Institute en het Clinical Research Center van de University of Texas Medical Branch (UTMB) in Galveston, Texas in de Verenigde Staten. Van augustus 1989 tot april 1990 was hij deeltijds aangesteld als toegevoegd docent bij de Vakgroep Fysiologie van Mens en Dier.

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