Consequences of different strategies of free amino acid supplementation to dietary proteins for physiological utilization.

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ABSTRACT

The efficiency of using free amino acids (AAs) as dietary constituent is sometimes lower than that of AAs derived from intact protein. The aim of the project was to evaluate dietary management conditions, which can determine the efficiency of utilization of crystalline AAs in animal diets or in clinical nutrition. The studies in this thesis were focused mainly on differences in short-term catabolism between protein bound and free AAs during the post prandial phase of a meal. The stable isotope technique was used in model studies with laboratory Wistar (WU) rats. In different experiments, so-called [$^{13}CO_2$]-breath test studies were used to compare the metabolic fate of free and protein-bound [1-^{13}C]-labeled AAs in a meal in various nutritional situations. The influence of free AA supplementation strategies on weight gain development was also studied. Moreover, protein and fat content in the liver and carcass were analyzed.

The results of the present study confirm literature that showed a higher post prandial catabolic losses of the tracer when dietary protein is replaced with crystalline AAs. In some, but not all situations a lower weight gain was observed for growing animals. Our results showed that short-term catabolic losses of endogenous leucine were modulated by exogenous AA supply. It increased or decreased depending on the adequacy of the dietary supply.

The common practice for free AA supplementation is to mix it with the deficient protein. In our study we showed that during the post prandial period the best utilization of methionine deficient protein did not occur when methionine supplement was given simultaneously with methionine deficient meal. The best utilisation occurred when there was a delay between the supply of deficient meal and free methionine. Therefore, introducing time interval of free amino acid supplementation to poor quality protein should be considered as a factor with potential to improve physiological utilization of dietary amino acids. A 1h time interval for free methionine supplementation influenced the weight gain, but differently depending on the protein level. Animals fed very deficient protein diet (5%) showed higher weight gain when supplementation of the deficient free methionine was provided with a 1h delay (1h interval). With protein deficient diets and less than 7.5 % protein we found fatty livers in our experiment. We concluded that post prandial AA oxidation influences the post absorptive AA catabolism. This does not always mean effect on growth. For growth in rats lysine deficiency is most limiting but it seems that even a 34% methionine deficiency below the NRC recommendation did not limit growth. We found that methionine deficiency influenced fat metabolism and from the literature we concluded that the mechanism probably works via choline and carnitine.

CONTENTS

General Introduction

In the current practice of animal production, low protein diets are often supplemented with crystalline amino acids to meet tabulated amino acid requirements. A supply of extra protein can have the same beneficial effect as addition of crystalline amino acids. However, overfeeding with protein increases N-excretion. On diary farms oversupply of protein by 6.6% results in a 16% increase in urinary N excretion (Jonker et al., 2002). Reducing the contamination of soils, water and air caused by excessive build-up of animal wastes is nowadays the priority of many nutritionists, land managers and lawmakers (D'Mello, 2003). Dietary crude protein can be reduced through the supplementation of synthetic amino acids. This approach reduces feed costs as well as N-excretion from pigs and poultry (Verstegen and Jongbloed, 2003; Kerr et al. 2003; Figueroa et al., 2002 & 2003; Kerr and Easter, 1995; Jongbloed et al., 1997; Nahm, 2002; Sutton et al., 1999). On the other hand, some studies have shown a tendency for pigs fed reduced crude protein (CP) AA-supplemented diets to have carcasses with more fat than pigs fed diets at recommended levels of CP (Kerr et al., 1995, Tuitoek et al., 1993; Schoenherr, 1992). The increased fatness in pigs fed low-CP, AA-supplemented diets may be partially due to more dietary energy being available for fat synthesis as a result of reduced energy expenditure for catabolisation of excess dietary protein (Kerr et al., 2003). One may expect differences in utilization of protein bound vs. free amino acids considering the differences in metabolism of both forms. In the case of free amino acids in a diet, they are absorbed immediately after arrival in the small intestine. Proteins require time-consuming proteolitic activity of the digestive tract to release the amino acids prior to absorption. Therefore, free amino acids reach the endogenous amino acid pool much earlier than amino acids bound in protein. There is no need for a physiological delay due to proteolysis. Thus, the time lag in absorption between free and protein-bound amino acids seems to make a difference in metabolism and in physiological utilization.

The exchange of free and protein-bound amino acids does not remain without physiological consequences. The nature of these consequences can be

different in relation to the nutritional situation. Complete exchange of protein-bound with free amino acids in animal diets does not give the same performance in terms of weight gain and carcass composition. Whole-body protein homeostasis is better supported by dietary bound than crystalline free amino acids (Daenzer et al., 2001). On short-term, molecular form of ingested nitrogen (free amino acids, peptides or proteins) and the type of dietary proteins affects amino acids absorption kinetics and degree of whole-body amino acid oxidation and utilization for protein synthesis (Metges et al., 2000; Collin-Vidal et al., 1994). The lower utilization of free amino acid appears due to the transient amino acid imbalance at the sites of metabolism after a more rapid absorption of the free AA compared to the protein-bound amino acids. This leads to the catabolism of the short-term surplus of free AA (Batterham et al., 1989).

Many nutritional factors like level of protein, protein/energy ratio, meal frequency or form of amino acids can influence dietary protein utilization. The commonly known fact is that not all meals consumed by animals and humans are nutritionally well balanced enough to meet the physiological needs of the body for protein synthesis. Because of different amino acid composition of various proteins, some of them are deficient in one or more amino acids. The amino acid that is in shortest supply in relation to need is termed ”the most limiting amino acid”. The limiting amino acid tends to be different in different proteins and for different species. The most often limiting amino acids from vegetable origin are Lys and/or Met (Mauro Di Pasquale, 1997). From the nutritional point of view, the protein amount along with amino acid composition and acute energy supply is most important for protein utilization. If these parameters are met, proper metabolism occurs. If there is a lack of energy in the diet, at least part of amino acids has to be oxidized. If some amino acids are deficient and protein unbalanced, the utilization for protein synthesis is limited and oxidative losses of amino acids increase.

The main point of concern is the synergism of the 20 precursor amino acids required during protein synthesis, especially in combination with a very

low level of free amino acids in the circulation. The body does not tolerate (too) high levels of amino acids. The pool of free amino acids is considered to hold only the 'currency' of nitrogen metabolism and comprises less than 1% of the total N in the body. This currency mainly originates from endogenous sources (protein turnover or *de novo* synthesis) and transiently from the feed as the exogenous source (Schreurs et al., 1992). As Millward (1992) stated, the body treats many indispensable amino acids as toxic metabolites. Good examples of their toxicity are some metabolic disorders such as maple syrup urine disease (MSUD) and phenyloketonuria (PKU). Because the degradation cannot proceed in those disorders, metabolites accumulate to toxic levels and cause disorders. Under normal conditions, amino acids have a short half-life in the circulation and therefore they are rapidly cleared. Amino acids are cleared by anabolic (protein synthesis) and catabolic (oxidation) processes. The anabolic pathway is rather constant or has at least a maximal value. Consequently, an irreversible catabolic process - oxidation (decarboxylation), clears any temporal oversupply of amino acids. This suggests that the initial metabolic handling of dietary amino acids immediately after a meal ('post prandial phase') is crucial for long-term utilization ('post absorptive phase').

Review by Shoveller et al. (2005) summarizes recent work on the importance of intestinal metabolism of dietary amino acids. For example, studies in infant piglets showed that the net portal absorption of several essential amino acids, including methionine and branched-chain amino acids (BCAAs), is considerably less than 100% of the dietary intake, ranging from 40 to 70% (Stoll et al., 1998). In addition, these studies suggest that as much as 30–60% of the dietary intake of leucine, lysine, and threonine is withdrawn in first-pass utilization by the gut and metabolized. Net intestinal utilization of methionine was substantial, consuming 52% of the dietary intake. Following in vivo studies with ^{13}C-labeled lysine and leucine showed that the gut represents a substantial proportion (20–30%) of whole-body oxidation of essential amino acids (Baracos, 2004; Schoor et al., 2002; van Goudoever et al., 2000; van der Schoor et al., 2001). The importance of the gut was also shown in piglet studies

designed to estimate the whole-body amino acid requirements by indicator amino acid oxidation in enterally and parenterally fed neonatal piglets (Shoveller et al., 2003a; Bertolo et al., 1998; Elango et al., 2002; Shoveller et al., 2003b). These studies indicated that the whole-body requirements for threonine, BCAAs, and methionine were significantly higher in enterally fed than in parenterally fed piglets. The methionine requirement was 30% greater in enterally fed than parenterally fed piglets fed methionine alone or in combination with excess cysteine (Shoveller et al., 2003a; Shoveller et al., 2003b). These data suggest that intestinal metabolism of dietary amino acids is nutritionally relevant.

On the other hand, microbial *de novo* amino acids synthesis also occurs in the gastrointestinal tract, and they can be absorbed (Metges, 1999 and 2000, Backes 2002). Irreversible loss of those amino acids from the gastrointestinal tract must also be considered. The microbial *de novo* amino acid synthesis and the gastrointestinal amino acid losses might as well be in balance with each other. The amount of net absorbed amino acids might therefore equal the dietary intake.

The studies in this project are mainly focused on differences in short term catabolism of protein bound and free amino acids during the post prandial phase of a meal. Stable isotope technique was used in model studies with laboratory Wistar (WU) rats. In different experiments, so-called [$^{13}CO_2$]-breath test studies were used to compare the metabolic fate of free and protein-bound [1-^{13}C]-labeled amino acids in a meal in various nutritional situations. [$^{13}CO_2$]-breath tests are simple, easily repeatable, and non-invasive. They are moreover very interesting as a functional test because the information obtained represents a dynamic evaluation rather than a static estimation (Evenepoel et al., 2000; Ghoos & Beaufrere, 1998; Stellaard & Geypens, 1998).

As breath test substrates in this project free and egg white bound [1-^{13}C]-leucine and free [1-^{13}C]-methionine were used.

Leucine was chosen as a tracer in [$^{13}CO_2$]-breath test technique, because this indispensable amino acid is used in the body mainly for protein synthesis. Leucine is a branched-chained amino acid (BCAA). Besides being substrate for

protein synthesis leucine serves as carbon precursor for ketone bodies and fat. Nitrogen from leucine is a source for synthesis of non-essential amino acids (alanine, glutamate, glutamine). The excess of leucine is subjected to energy metabolism (Reeds, Davis, 1999). The first step of leucine degradation is the reversible transamination to α-ketoisocaproate (KIC). The amino group is transferred to 2-oxoglutarate, which forms glutamate. KIC is the immediate precursor for leucine decarboxylation, which is the second step of degradation. This is an irreversible oxidative reaction. CO_2 from the carboxyl group is released in this step (Stryer, 1999). CO_2 excreted due to this reaction can be measured if leucine was labeled with stable isotope of ^{13}C in this position. The chain of those reactions leads to formation of acetyl CoA and acetoacetate. The former may join the citric acid cycle or fat synthesis (Stryer, 1999). Figure 1 presents simple schema of leucine metabolism.

Because leucine is an indispensable amino acid, and has no other nor protein, nor oxidative functions, often is used as marker in studies on protein metabolism. ^{13}C-leucine technique for measuring whole-body protein kinetics is the most commonly used precursor method (Fouillet et al., 2002).

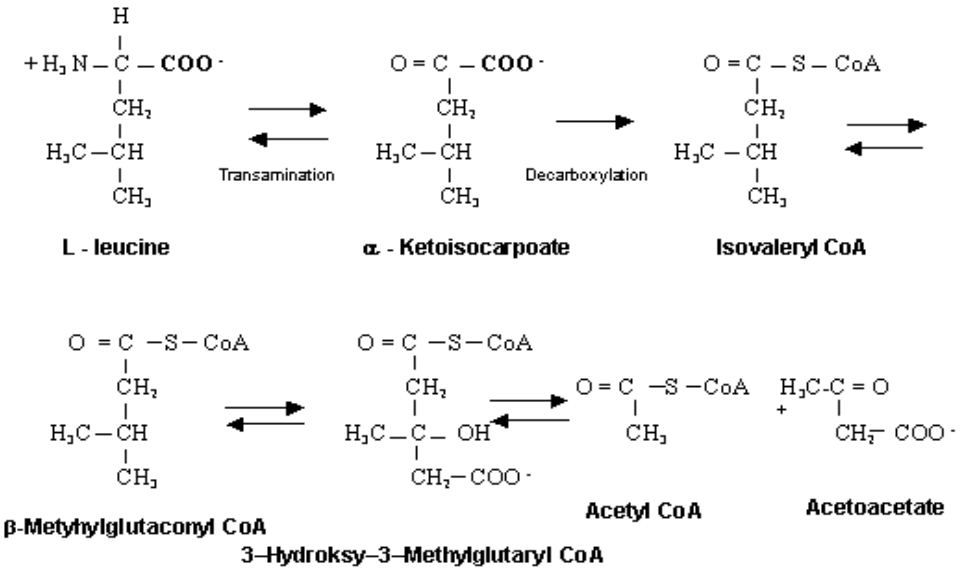

Figure 1: Schema of leucine metabolism.

Leucine is now used for more than 20 years to assess whole body protein metabolism. It was proved by Motil et al. (1981), Reeds and Davis (1999) that leucine may be the most generally useful label for tracer studies of both whole-body and muscle protein synthesis.

In short, leucine not used for protein synthesis is decarboxylated. Therefore, the decarboxylated part allows assessing the part of amino acids that is lost for protein metabolism (Reeds and Davis, 1999).

Methionine deficient diets were studied because methionine along with lysine is the most common limiting amino acid (as mentioned before). The choice for methionine was based on previous studies with humans in our laboratory. Three exogenous amino acids (leucine, lysine and methionine) in egg white bound and free form were used as substrates in breath test studies. Labeled methionine showed highest oxidative losses and most pronounced differences between bound and free form with room for reduction. We used amino acids, which can have different fates in the body. To study the time lag in absorption of free and protein-derived amino acids, different time intervals for free methionine supplementation was used.

The aim of the project was to get a better view on dietary conditions that might improve the use of crystalline amino acids in animal diets or in clinical nutrition under practical conditions.

The general scope of the research in the present dissertation was to investigate possibilities to improve free amino acid supplementation of poor quality proteins for better physiological utilization of amino acids. The more specific objectives were:

► to determine whether ingestion of free or protein bound amino acids influences short-term catabolism of endogenous amino acids during the post prandial phase of a meal. For background purpose, this study was also meant to confirm the influence of molecular form of ingested nitrogen on weight gain and dietary amino acid short-term catabolic losses when bound vs. free amino acid were the source of protein in a diet. (chapter 1)

► To investigate whether time interval for free methionine supplementation of 50% methionine deficient dietary protein improves post prandial amino acid utilization measured in breath tests with egg white bound [1-^{13}C]-leucine and free [1-^{13}C]-methionine. Moreover, this study also examined whether any adaptation to the same feeding pattern can be seen after 3 weeks of experimental feeding (chapter 2).

► To investigate influence of time interval (0 vs. 1 h) for free methionine supplementation of 50% methionine deficient dietary proteins on short-term post prandial amino acid oxidation on diets with different level of protein (5, 7.5, 10 & 13.8%). In addition in this study, possible influence of time interval, for free methionine supplementation at various protein levels, on body composition was examined with respect to fat and protein content in dry matter of body and the livers (chapter 3).

► To study the impact of Lys and/or Met deficiency on body weight gain and carcass and liver composition of growing rats. Specific functions and interactions of methionine and lysine were analysed (chapter 4).

Finally, all findings are discussed in General Discussion and possible physiological and methodological explanations are given.

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Chapter 1

Short term catabolism of endogenous amino acids in rats is enhanced by exogenous amino acids supply. [$^{13}CO_2$]-breath test study with L-[1-^{13}C]-leucine.

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ABSTRACT

The influence of dietary leucine bound in egg white protein or the corresponding free amino acid (FAA) mixture on short-term catabolism of leucine from the endogenous amino acid (AA) pool was investigated. Male growing rats were adapted for 8 days to one of two isoenergetic, isonitrogenous diets (2.1 g N /100 g) containing either egg white bound amino acids (BAA) (diet1) or FAA (diet 2). On day 9, the rats received an intraperitonial (IP) injection of leucine (labeled or non-labeled) and were [$^{13}CO_2$]-breath tested in one of seven nutritional conditions: three for animals on the BAA diet and four for animals on the FAA diet. In all conditions six animals were breath tested with [1-^{13}C]-leucine as a substrate. The substrate was: given as intrinsic part of a meal (bound or free), IP injected in absence of a meal, IP injected prior to ingestion of a meal. In addition the animals on the FAA diet were also breath tested after IP injection of [1-^{13}C]-leucine prior to ingestion of a FAA meal without leucine (FAA-Leu). Weight gain during 8 days on experimental diets was lower ($P < 0.05$) in rats given the FAA diet compared to BAA diet, indicating long term effect of the use of free amino acids in the diet. Short-term catabolism of dietary free leucine was twice as high as for egg white bound [1-^{13}C]-leucine ($P<0.05$). The short-term catabolism of labeled leucine in the endogenous AA pool in the post-prandial phase was influenced by the meal and its characteristics. In the case of treatment with FAA meal without leucine, catabolism of endogenous leucine was decreased ($P < 0.05$) to compensate leucine deficiency of the meal. In the case of non-deficient meal there is an extra loss of both exogenous and endogenous AAs. There is an increase in catabolism of endogenous AAs in addition to loss of exogenous AAs. We conclude that short term catabolism of leucine from endogenous AA pool is sensitive to exogenous amino acids supply. Catabolism of endogenous leucine is driven by short term availability. It is more pronounced when crystalline vs. bound AAs are ingested and reduced when leucine is absent in the diet.

Key Words: dietary amino acids, egg white bound [1-^{13}C]-leucine, free [1-^{13}C]-leucine, intraperitoneal injection, endogenous amino acids, [$^{13}CO_2$]-breath test

INTRODUCTION

Studies by Bujko et al. (to be published) show that the [$^{13}CO_2$]-breath test approach is a sensitive tool to establish the catabolism of dietary leucine shortly after a meal. A comparison of free and egg white derived L-[1-^{13}C]-leucine (hereinafter referred to as "tracer") indicated that the digestive tract fractionates free and bound leucine from the diet with respect to the timing of their metabolic availability. This fractionation has quantitative consequences for their post prandial catabolic losses.

From a nutritional point of view, we assume that the body converts exogenous AAs to endogenous AAs. The question is to which extent catabolism of endogenous AAs follows catabolism of exogenous AAs, measured by the [$^{13}CO_2$]-breath test. Bujko et al. (to be published) found a clear difference between the catabolism of free and egg white bound [1-^{13}C]-leucine but the simultaneous response in catabolism of endogenous AAs was not examined. Therefore, this study with rats investigated whether catabolism of AAs from endogenous AA pool is affected by exogenous AA supply (free or protein bound). At first the short-term catabolism of dietary AAs was measured for the conditions of this study. In the second set of breath tests the endogenous AA pool was labeled by an IP injection of [1-^{13}C]-leucine to study the short-term catabolism of endogenous leucine under various nutritional conditions. Seven nutritional conditions were tested: three for animals on the BAA diet and four for animals on the FAA diet. All animals received intraperitoneal injection of leucine (labeled or non-labeled). In all conditions 6 animals were breath tested with [1-^{13}C]-leucine as a substrate. The substrate was: given as intrinsic part of a meal (bound or free), IP injected in absence of a meal, IP injected prior to ingestion of a meal. In addition the animals on the FAA diet were also breath tested after

IP injection of [1-^{13}C]-leucine prior to ingestion of a FAA meal without leucine (FAA-Leu).

Breath test measurements were continued for 7.5 hours after a meal.

MATERIALS AND METHODS

Animals, housing, feeding and diets.

This study was performed with 36 male, 7 week old Wistar (WU) rats (Harlan, Horst, The Netherlands) with an initial weight of 200-225g. The rats were housed individually in macrolon cages (38 x 26 x 14 cm) in the animal facilities at controlled temperature (21^{o}C) and relative humidity (70%). The daily light schedule was divided to 8 hours of darkness and 16 hours of light. For convenience of the feeding the dark period was set during day time (09.00 - 17.00). Water was available 'ad libitum'. The animals were first familiarized with new environment for 20 days before the start of dietary treatments. During this pre-conditioning period commercial rat chow (Teklad Global Rodent Diet; Harlan, The Netherlands) was provided 'ad libitum' as 2 mm pellets.

After the pre-conditioning period, the animals were assigned to one of the two experimental diets. The two diets were semi-synthetic, iso-nitrogenous and isoenergetic, with either egg white bound amino acids (BAA) or free amino acids (FAA). The BAA animals (n = 18) received a diet with 13.2 % egg white as the only protein source. Free lysine and methionine added to this diet fulfilled the tabulated requirements of amino acid pattern for growing rats published by National Research Council (1995). The FAA animals (n=18) received a diet with the same composition but the protein source was replaced by a mixture of free amino acids. The mixture was simulating the egg white protein according to Evenepoel (1997). During breath test, meals with bound amino acids (BAA), free amino acids (FAA) and free amino acids without leucine (FAA-Leu) were tested. The animals on BAA diet could not be tested in absence of leucine in the meal because the egg white protein cannot be devoided of leucine. For the FAA-Leu diet, the AA pattern was as in table 1, but without leucine.

During the 20 day pre-conditioning period the feed intake was 'ad libitum' until the start of experimental diets. Thereafter, the feeding was restricted to two 30-min periods at the beginning and at the end of darkness (9:00 and 16:30, respectively). During the experimental period of 8 days rats were given a fixed amount of 5 g in the morning and 10 g in the evening meal. The morning meal was smaller in order to ensure it would be eaten completely within the first 15 minutes collection period of the [$^{13}CO_2$]-breath test. After a few days, rats ate the morning meal within 5 minutes. Experimental feed was given as porridge to prevent spilling of tracer to be included in the test meals. The porridge was a mixture of dry food and water (2:1).

Table 1. Composition of the experimental diets (g/kg). The two experimental diets differed in crude protein fraction. The diet with protein contained egg white protein with minor amounts of free lysine and methionine to fulfil the tabulated requirements for growing rats (NRC, 1995). The diet with only free amino acids contained a mixture of amino acids simulating egg white pattern according to Evenepoel et al (1997) also with minor amounts of free Lys and Met to meet the requirements. The diets were iso-nitrogenous and iso-energetic.

Ingredients	Free amino acid meal (grams)	Bound amino acid meal (grams)
Dextrose	50,0	50,0
Sugar (Saccharose)	135,2	139,6
Wheat starch	475,0	475,0
Soya oil	50,0	50,0
Arbocel cellulose	75,0	75,0
Vit. Premix	12,0	12,0
Min. Premix	10,0	10,0
Egg white protein	-	132,0
CaCO3	19,0	19,0
MgO	1,5	1,5
NaH2PO4.2H2O	6,5	6,5
KH2PO4	14,0	14,0
KHCO3	10,0	10,0
NaCl	0,5	0,5
Free Amino Acids		
L-isoleucine	7,4	-
L-leucine	10,8	-
L-lysine HCL	11,8	2,0
DL-methionine	7,5	2,9
L-cysteine	2,4	-
L-fenylalanine	7,7	-
L-tyrosine	5,3	-
L-Threonine	6,3	
L- tryptophane	2,4	-
L-valine	10,3	-
L-arginine	7,1	-
L-histidine HCL	4,8	-
L-alanine	7,7	-
L-aspartate	14,4	-
L-glutamate	16,1	-
Glycine	4,2	-
L-proline	5,3	-
L-serine	9,8	-

Breath test conditions (scheme 1):

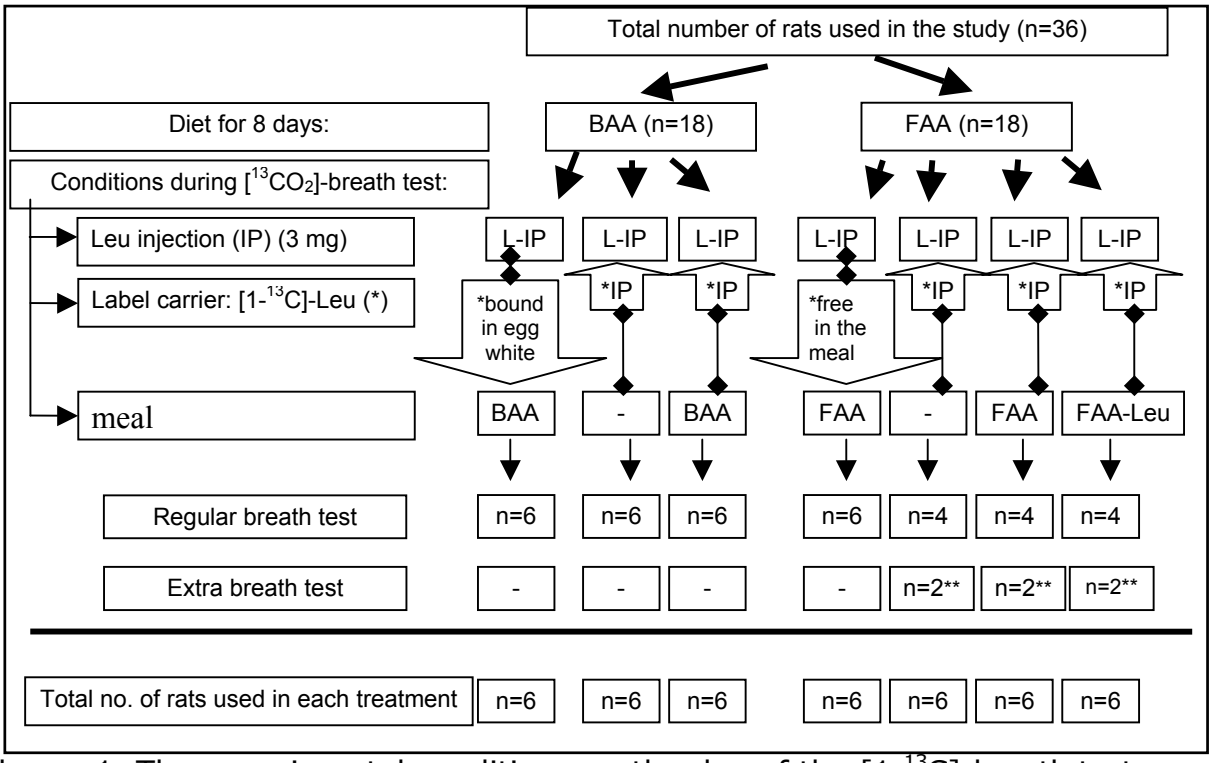
Animals were breath tested after 8 days on the experimental diets (BAA vs. FAA). Each test was performed with six rats.

Rats conditioned on the BAA diet were breath tested with labeled [1-^{13}C]-leucine:

- incorporated in egg white protein as intrinsic part of the BAA meal (n=6),
- IP injected in absence of the BAA meal (n=6),
- IP injected prior to ingestion of the BAA meal (n=6).

Rats conditioned on the FAA diet were breath tested with labeled free [1-^{13}C]-leucine:

- as intrinsic part of the FAA meal (n=6),
- IP injected in absence of the FAA meal (n=6),
- IP injected prior to ingestion of the FAA meal (n=6),
- IP injected prior to ingestion of a FAA meal without leucine (FAA-Leu) (n=6).



Scheme 1. The experimental conditions on the day of the [1-^{13}C]-breath test.

BAA- bound amino acid diet; FAA- free amino acid diet; FAA-Leu- free amino acid diet without leucine; n- number of rats; L-IP- intraperitoneal injection of leucine,

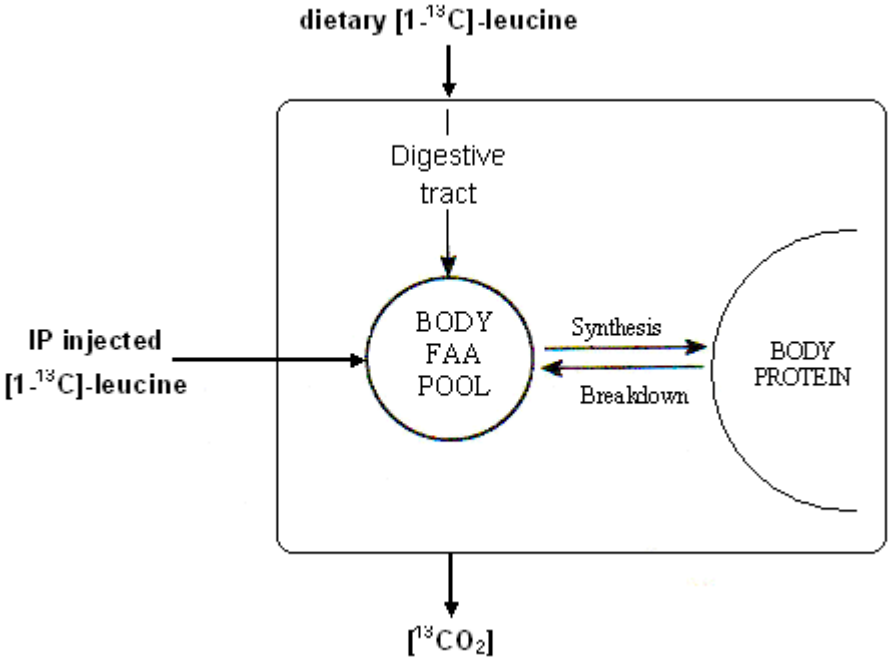
*- label ([1-^{13}C]-leucine) carrier

** On the 2nd breath test day there were animals used second time, the animals had 1 day rest (therefore they were conditioned 10 instead of 8 days on FAA diet). They were used for different treatment than on 1st day to avoid carry on effect of the treatment.

In the first set of breath tests (label "in the meals"), the animals on the BAA and FAA diet underwent the breath test measurements with L-[1-^{13}C]-leucine either egg white bound or in free form present in the test meal, respectively. The labeled egg white protein was produced by feeding L-[1-^{13}C]-leucine to laying hens according to the method of Evenepoel et al (1997). For more details about labeling of breath test meals see the paragraph on 'Breath test substrates'.

In the second set of breath tests (label "in endogenous AA pool"), the label was intraperitoneally (IP) injected to study short-term catabolism of the tracer from the endogenous AA pool. IP injected amino acids are supposed to enter endogenous

AA pool (Schreurs et al., 1992). Therefore, we assume that the IP injected amino acid faces the same fate as the body derived amino acid in the FAA pool. Scheme 2 shows a model of the [$^{13}CO_2$]-breath test principles for better understanding of this experiment. Expiration of [$^{13}CO_2$] derived from [1-^{13}C]-leucine reflects irreversible decarboxylation of the tracer and indicates that leucine is wasted for protein metabolism of the body.



Scheme 2. Model of [$^{13}CO_2$]-breath test principles

At the end of the experiment, the animals were sacrificed with an O_2/CO_2 gas mixture (ratio 1:2). The Committee for Animal Welfare of Wageningen University approved the present study.

Measurements

Body weight development

All rats were weighed daily prior to their morning meal at 09.00 hour. The weighing procedure made the rats familiar with being handled. The body weight of rats was followed to check the influence of the dietary treatment on the weight gain of the animals.

[$^{13}CO_2$]-breath test

◇ General aspects of the procedure

The [$^{13}CO_2$]-breath test procedure performed according to Bujko et al. (to be published) was used in this study to follow the dynamics of L-[1-^{13}C]-leucine present in the diet or in the endogenous AA pool during the first hours after a meal.

By introducing labeled amino acid as part of a meal short term catabolism of food-derived amino acids was measured. By introducing labeled amino acid directly in the body AA pool by an intraperitoneal injection short term catabolism of amino acids from the endogenous AA pool was measured.

The expiration of [$^{13}CO_2$] was measured during successive 15-min periods for the first 3 hours after the tracer ingestion or injection. Thereafter, the samples were taken every 30-min for the next 4.5 hours. In total the sampling period covered 7.5 hours after introduction of the [^{13}C]-labeled substrate.

The appearance of [$^{13}CO_2$] in the breath reflects metabolic fate and the kinetics of the [^{13}C]-labeled leucine. The kinetics of rate expiration of the label is expressed as percentage of the initial dose of substrate per hour (% dose / h). The total capacity of the rat to expire the label is determined as the cumulative recovery of label during the entire 7.5 h period of breath collection. The cumulative values are expressed as percentage of the dose expired in 7.5 hours (% dose / 7.5h).

◇ Breath test substrates

The labeled substrate used in this study was L-[1-^{13}C]-leucine (chemical purity > 99%, isotopic enrichment > 99%, ARC, Amsterdam, The Netherlands), which was either ingested as intrinsic part of a test meal or applied to endogenous body pool by an intraperitoneal injection. In all cases a well known amount of about 3 mg of the tracer was used as breath test substrate. This amount has been found to be about the threshold value of a tracer dose that does not increase the level of oxidation in itself. At the same time, this amount

of a tracer is the minimal amount that allows the [^{13}C]-enrichment of CO_2 to be measured with IRMS analyzer (Schreurs and Koopmanschap, 1996). In the case of IP injection 200 μl of water was used to dissolve the substrate. If the labeled AA was part of the meal, animals got IP injection with 3 mg of non-labeled leucine to make the experiments comparable.

In the case of exogenous labeling, L-[1-^{13}C]-leucine was used in two dietary forms: free or incorporated in egg white protein. It is essential that the labeled substrate is an intrinsic part of the meal to avoid pharmacological effects of the substrate on the catabolic response (Schreurs & Koopmanschap, 1996).

When given in the meal approximately 5% of the non-labeled leucine was replaced by the tracer. With these amounts of test substrate the [^{13}C]-enrichment of expired CO_2 could be determined properly. In the case of the FAA-diet, 3.0 mg of the tracer replaced non-labeled leucine of the test meal. In the BAA-diet about 50 mg of the labeled egg white protein (ranging from 1.30 - 1.40 Atom %) was used in the test meal. This amount of labeled egg white also contained about 3 mg of [1-^{13}C]-leucine.

In the case of endogenous labeling, the label was introduced in the endogenous AA pool by an intraperitoneal injection (IP).

◇ Breath test procedure and sampling

On day 9 after the start of the experimental diets (for 6 rats again on day 11) the [$^{13}CO_2$]-breath tests were performed. The breath test procedure started at 08.45 hour, 15-min prior to the regular feeding time. This first 15-min period was used to collect a blank sample for the natural [^{13}C]-enrichment of expired air for each individual rat. If the animals were used twice, the background value had to be measured because of the presence of the label from the breath test on the previous day. In practice, this aspect was negligible. Just before the feeding time (09.00 hour) the animals received an IP injection of 3.0 mg of leucine: L-[1-^{13}C]-leucine (those animals tested for catabolism of leucine from endogenous AA pool) or non-labeled leucine (those animals that received the

label in the meal and were tested for catabolism of exogenous leucine). Immediately after the injection, the designed meals were provided.

In order to sample the expired air adequately, rats were placed individually in an airtight macrolon cage (20 x 16 x 14 cm, ± 4 litre) bedded with sawdust and with free access to water. A sampling period of at least 15-min was required to build up a level of CO_2 adequate for analysis of the [^{13}C]-enrichment of CO_2 (3 - 5 % v / v). At the end of each sampling period, 50 ml of air was collected with a syringe through a lid on the cage. The syringe was emptied in a 10 ml exetainer tube (Labco, High Wycombe, UK). The tubes were stored at room temperature until analysis. After each 15 or 30 min sampling period, the animals were transferred to a fresh cage for the next 15 or 30-min collection period.

◇ Sample analysis

The air samples were analysed by IRMS (Isotope Ratio Mass Spectrometry) for the [^{13}C]-enrichment of CO_2, expressed as Atom% (^{13}C/total C), with an accuracy of ± 0.0005 %. The tubes with the air samples were directly placed in the auto sampler of the breath device linked to the IRMS-analyser (Delta C; Finnigan MAT, Bremen, Germany).

◇ CO_2 - production

The absolute amount of [^{13}C] expired can be calculated only when in addition to the [^{13}C]-enrichment value, the total amount of [C] expired as CO_2 is known.

Values for the CO_2-production were checked four times in separate trials (n=2 for each dietary animals) for comparison with total CO_2-production measurements performed as described by Bujko et al. (to be published).

As discussed in the study of Bujko et al. the variation in the pattern and the level of total CO_2 production vs. time was negligible compared to the differences in [^{13}C]-enrichment of the air expired by animals on both diets. Substantial changes in [^{13}C]-enrichment of expired CO_2 can be expected because the breath

test has no steady state for the tracer, after the bolus application of label. Therefore, the fluctuation in total CO_2 production is relatively small and can only have a minor impact on the conclusions of this study. In line with Bujko et al., we used a constant value for the CO_2-production of each single rat. The values were based on the measured value of 7.5 ml CO_2 / min / 0.26 kg body weight and corrected for metabolic weight $(W)^{0.75}$.

◇ Calculation of label recovery

In this study, the expired amount of label as a percentage of the applied amount was calculated after Bujko et al. (to be published) as follows. For each air sample the [^{13}C]-enrichment was determined by IRMS-analysis. The Atom%-excess value was determined as the difference between the [^{13}C]-enrichment of the sample and the blank (pre-meal value). The absolute amount of [^{13}C] expired was calculated using the Atom%-excess value and the total amount of [C] expired as CO_2 during the same period of sampling. As explained above we used the mean value for the production of CO_2 for all animals after correction for metabolic body weight. This mean value about 7.5 ml / min was multiplied by duration of sampling period (15 and 30 min), divided by 22.4 ml / mmol (molar volume) and multiplied by 12 (atomic weight of carbon). The outcome is that the rats expired 60 mg of Carbon during each 15-min and 120 mg during each 30-min sampling period. The absolute amount of [^{13}C] expired is then calculated as the Atom%-excess value of the amount of Carbon expired. This absolute amount of [^{13}C] is expressed as percentage of the amount of applied [^{13}C]. In case of 3 mg L-[1-^{13}C]-leucine 0.30 mg (13/132 x 3) was applied as [^{13}C]. The rate of expiration was expressed as % dose / h. The cumulative recovery was expressed as percentage of dose / 7.5h and was calculated by adding up the absolute amounts expired in the subsequent sampling periods.

Statistics

For statistical analysis SPSS for Windows was used (12.0.1). The values are expressed as mean ± SD. The data was analysed by one-way analysis of variance (one-way ANOVA). In case the data did not have normal distribution the median analysis using the Kruskal-Wallis test was performed. For significant influence, post hoc testing was used with Bonferroni testing. Differences were considered significant when $p<0.05$.

RESULTS

Body weight development

During the pre-conditioning period (20 days) on 'ad libitum' commercial feed rats gained on average 3 grams a day. After overnight fasting and the first day on experimental diets, the mean body weight of all animals dropped because of restricted feeding to 15 g/day. After 1 day on experimental diets the weight was not significantly different between animals of the two diets: 272 ± 3 g (BAA) and 266 ± 3 (FAA). The median analysis using the Kruskal-Wallis test showed that the mean body weight of animals on the FAA diet (270 ± 3g) was significantly lower ($p<0.05$) compared to the animals on the BAA diet (284 ± 3g) after 8 days. The mean weight gain during 8 days was also significantly lower for the FAA animals (4 ± 2g) compared to the BAA animals (12 ± 2 g).

[$^{13}CO_2$] -breath test at day 9 on the experimental diet

I. Recovery of [$^{13}CO_2$] from measurements with exogenous substrate.

◇ BAA-diet breath tested with labeled egg-white protein.

The expiration of ingested label as [$^{13}CO_2$], started within the first 15-min collection period (figure 1). The expiration rate gradually increased to 2.3 % of the dose / h after 90 min. Between 90 and 300 minutes the values of the expiration rate were not significantly different but a biphasic pattern could be recognized. The highest rate of expiration (2.7% of the dose/h) was measured after 300 minutes. Thereafter the value declined. At the end of the collection period

(450 minutes) the expiration rate was approximately 1.0 % dose / h. The cumulative recovery of label for the animals on BAA diet was 15.2% dose / 7.5h.

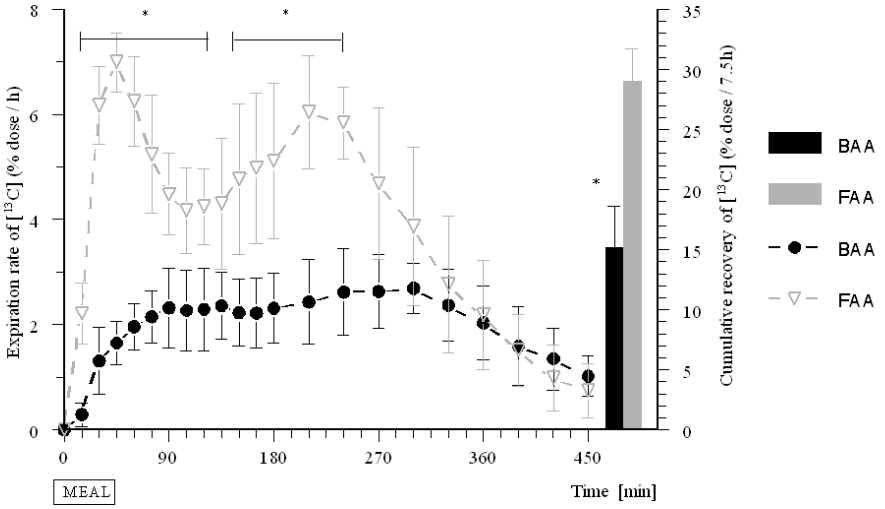


Figure 1: Expiration rate (lines) of label as [$^{13}CO_2$] vs. time, expressed as % dose / h of ingested L-[1-^{13}C]-leucine administered in a test meal as bound to egg white protein (BAA) or in free form (FAA). Breath tests were performed at day 9 after the start of the experimental diets. Corresponding bars represent cumulative recovery of label expressed as % dose / 7.5h.

** indicates significant differences between FAA and BAA (p < 0.05).*

◇ FAA-diet breath tested with labeled free leucine.

The expiration rate of ingested label as [$^{13}CO_2$] by the animals on FAA-diet (free amino acids only) increased faster and reached a higher level than for the animals on BAA-diet. The maximal expiration of 7.0% of the dose/h was reached after 45 minutes. The value decreased gradually to a value of 4.2% dose / h after 105 min (figure 1). Thereafter there was again an increase in expiration rate of the label until a second peak value was reached of 6.0 % after 210 minutes. From 210 min after the start of the meal onwards, a gradual decrease in the expiration rate was noticed. At the end of the collection period (450 min) the expiration rate of BAA and FAA were at the same level (± 1%). The values for the rate of expiration for the FAA-diet differed significantly from the BAA-diet

between 15 through 120 min and between 150 through 240 minutes after ingestion of the test meals. The cumulative recovery of label of 29.0 ± 2.7 % dose / 7.5h for the FAA-diet is different from the 15.2 ± 3.4 % dose / 7.5h for the BAA-diet ($p < 0.05$).

II. Recovery of [$^{13}CO_2$] from measurements with endogenous substrate

Animals on BAA diet were breath tested with intraperitoneally injected labeled leucine:

◇ in absence of a meal

The expiration rate of label as [$^{13}CO_2$] started within the first 15-min collection period (figure 2). The expiration rate of [$^{13}CO_2$] increased quickly and reached a maximum of 35.2 % of the dose / h after 30 min from the tracer injection. Within 15 min it decreased to half of the maximum value. The decrease in the expiration rate continued to less than 1 % of the dose / h after about 165 min. At the end of the measurement (after 7.5 hours) the expiration rate had a value of 0.5 % of the dose / h. The cumulative recovery of label for the animals on BAA – diet in absence of the meal on breath test day was 30.2% of the dose / 7.5h.

◇ in presence of a BAA meal

The expiration rate of intraperitoneally injected label as [$^{13}CO_2$] in presence of a BAA-meal started (like in the group without a meal) within the first 15-min collection period (figure 2). The expiration rate increased as quickly but reached higher maximum of 39.9% of the dose / h after 30 min compared to the results in the absence of a meal. Thereafter the expiration rate decreased rapidly and went down to less than 1% of the dose / hour after about 360 minutes. At the end of the measurement (after 7.5 hours) the expiration rate had a value of 0.4 % of the dose / h.

The cumulative recovery of label of 34.1% dose / 7.5h in the presence of the BAA meal was not significantly higher than the 30.2 % dose / 7.5h in the absence of the BAA meal.

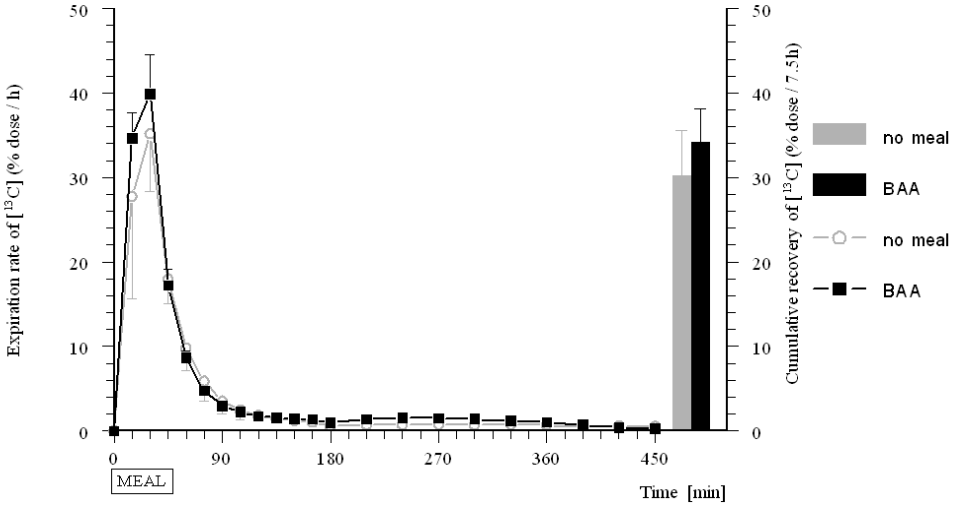


Figure 2: Expiration rate (lines) of label as [$^{13}CO_2$] expressed as % dose / h of intraperitoneally injected (IP) L-[1-^{13}C]-leucine together with bound amino acid meal (BAA) and without a meal. Breath tests were performed at day 9 after the start of the BAA diet. Corresponding bars represent cumulative recovery of label expressed as % dose / 7.5h.

FAA-diet animals breath tested with intraperitoneally injected labeled leucine:

◇ in absence of a meal

The expiration rate of intraperitoneally injected label as [$^{13}CO_2$] by the animals of FAA-diet in absence of a meal, started within the first 15-min collection period (figure 3). The expiration rate increased quickly and reached its maximum of 35.6% of the dose / h after 30 min from the tracer injection. Thereafter it quickly decreased and went down to less than 1 % of the dose / h after about 420 min. At the end of the experiment (after 7.5 hours) the expiration rate was 0.9 ± 0.3 % of the dose / h. The mean cumulative label recovery in the FAA animals in absence of a meal was 33.7% of the dose / 7.5h. This did not significantly differ from the animals conditioned on BAA diet.

◇ in presence of a FAA meal

The expiration rate of intraperitoneally injected label as [$^{13}CO_2$] by the animals on FAA-diet during a FAA meal, started also within the first 15-min collection period (figure 3). The expiration rate increased quickly but reached a higher maximum (42.3% of the dose / h) than in the absence of a meal. The peak value was reached in both cases after 30 min from the tracer injection. In the next 15 minutes it rapidly decreased to the value of 28.3% of the dose / h. Thereafter there was further decrease in the expiration rate of the tracer accompanied by complete FAA meal to less than 1 % of the dose / h after about 330 min. There was significant difference in the tracer recovery between animals in the two nutritional situations (FAA meal vs. no meal) from 30 through 120 minutes. At the end of the experiment (after 7.5 hours) the expiration rate was 0.4 ± 0.4% of the dose / h. The mean cumulative label recovery in the FAA animals in presence of a meal was 39.1% of the dose / 7.5h. This did differ ($p<0.05$) from the animals conditioned on BAA diet but in absence of the meal on breath test day.

◇ in presence of a FAA meal without leucine (FAA-Leu)

The expiration rate of intraperitoneally injected label as [$^{13}CO_2$] by the animals on FAA-diet that on the measurement day received FAA meal without leucine (FAA-Leu), started also within the first 15-min collection period (figure 3). The expiration rate increased quickly and reached lower maximum (28.6% of the dose / h) compared to both nutritional situations described above, (significantly lower than after the FAA meal). After 45 min. the expiration rate had decreased to a value of 11.0 % of the dose / h. Thereafter there was further decrease in the expiration rate in the FAA-Leu group to less than 1 % of the dose / h after about 120 min. At the end of the experiment (after 7.5 hours) the expiration rate was 0.3 ± 0.3% of the dose / h.

The cumulative recovery of ^{13}C in the presence of FAA meal without leucine (22.9 % of the dose / 7.5 h) was significantly lower than in both absence and presence of

the FAA meal. It differs ($p<0.05$) also from the animals conditioned on the BAA diet, both in the absence and presence of the meal on breath test day.

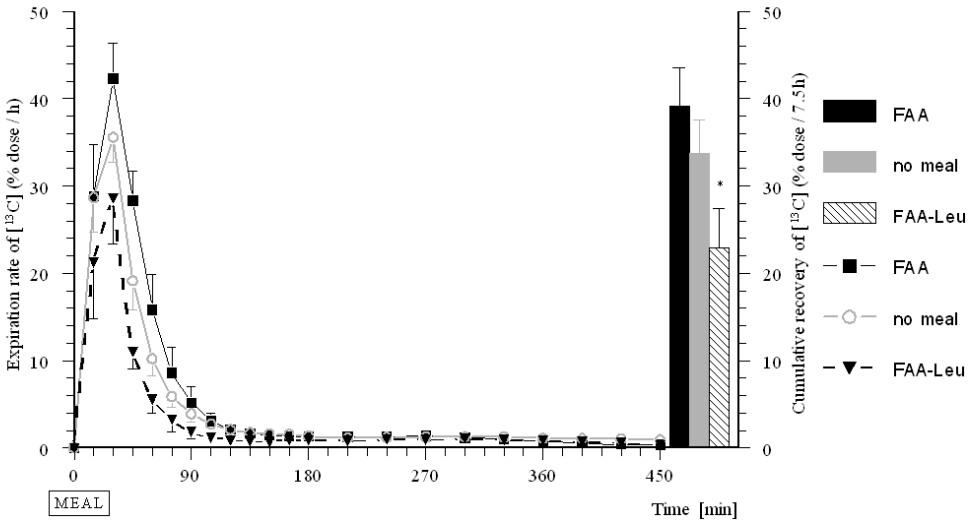


Figure 3: Expiration rate (lines) of label as [$^{13}CO_2$] expressed as % dose / h of intraperitoneally injected L-[1-^{13}C]-leucine together with: free amino acid meal (FAA), in absence of a meal, free amino acid meal without leucine (FAA-Leu). Breath tests were performed at day 9 after the start of the FAA diet. Corresponding bars represent cumulative recovery of label expressed as % dose / 7.5h.

** indicates significant differences ($p < 0.05$).*

DISCUSSION

The objective of the present study was to investigate to what extent the catabolism of endogenous AAs follows catabolism of exogenous AAs, measured by the [$^{13}CO_2$]-breath test. Body weight gain of rats fed either egg white protein or free amino acid diet of the same amino acid pattern was followed as well.

Development of body weight

During 8 days of the experimental period, the body weight gain of rats on the FAA-diet (4 ± 2g) was significantly lower than rats on the BAA-diet (12 ± 2 g). This difference can only be a direct or indirect effect of the use of free amino acids in the diet, as that was the only difference between the two groups. Our

results confirm the earlier findings of lower body weight gain of rats and piglets on free amino acid mixtures compared to protein diets (Daenzer M et al. 2001, Poullain M. G. et al.,1989; Officer D. I. et al., 1997; Trocki O. et al., 1986). The effect on body weight gain, however, is not observed when low-protein diets are supplemented with crystalline amino acid(s) (Kerr et al., 1995, Easter and Baker, 1980, Russell et al., 1983)

[$^{13}CO_2$]-breath test

A [$^{13}CO_2$]-breath test is a simple and non-invasive method and is known as a helpful tool to study various metabolic processes (Raguso et al., 1999; Reeds & Davis, 1999). The [$^{13}CO_2$]-breath test approach has been used in our laboratory (Bujko et al. to be published) as a method to study short-term dynamics of dietary amino acids. In the present study, in one part of the experiment the appearance of [$^{13}CO_2$] in the breath was measured during 7.5h after ingestion of a test meal containing exogenous L-[1-^{13}C]-leucine (egg white bound or free). In another part of the experiment, the same was measured after intraperitoneal injection of the tracer. In the first case (exogenous label), the appearance of [$^{13}CO_2$] in the breath reflects the overall kinetics of dietary components through processes of oral ingestion, gastric emptying, digestion, absorption, distribution in the body, decarboxylation and expiration as [$^{13}CO_2$]. The IP injected labeled amino acids bypass the digestive tract and enter the endogenous amino acid pool via the blood (Schreurs et al, 1992). In this case, the appearance of [$^{13}CO_2$] in the breath shows decarboxylation of leucine from the endogenous AA pool.

In the study of Bujko et al. (to be published), short term catabolism of dietary amino acid in free form was much higher than when dietary amino acids were bound in egg white protein. This raises the question whether or not the higher short term catabolic losses of free amino acids are an extra loss from a nutritional point of view or that there might be a compensation by a reduced catabolism of endogenous AAs. In this study, the interaction between metabolism of AAs from the body

and diet was investigated. Exogenous [1-^{13}C]-leucine was supplied in a FAA and in a BAA meal. The BAA meal is considered as the reference meal since food contains mainly BAAs.

exogenous label

The curves in figure 1 show that the [^{13}C] recovery rate for the FAA meal exceeds the recovery rate of that of a BAA meal. The differences are significant between 15 and 240 min. Also the cumulative recovery of label was significantly different (29.0% vs. 15.2%, for FAA and BAA respectively). This implies that the short-term catabolic loss of dietary AA is always substantial but nearly twice as high for FAA compared to BAA. This data confirms the results of Bujko et al. (to be published). The conclusion coming from both studies is that there is higher catabolism of free dietary leucine than protein bound leucine in the post prandial phase. Nevertheless, the pattern of ^{13}C expiration rate between the two studies was different probably because of the use of different diets with different protein levels. In addition, the meal size in this study was smaller. Therefore, a lower amount of leucine was ingested with the meal.

Other studies also report different post prandial leucine kinetics (Collin-Vidal C. et al., 1994; Metges et al., 2000) when leucine is ingested in different dietary forms. Studies with crystalline lysine and threonine vs. protein bound lysine and threonine showed also a more rapid absorption of free amino acids (Yen et al., 2004). The study by Daenzer et al., 2001 concluded that whole-body protein homeostasis is better supported by dietary casein-bound than crystalline free amino acids. They suggested that differences in kinetics of amino acid absorption and consequently post absorptive utilization might be an important explanation for lower efficiency of free amino acid-based diets compared to diets containing intact proteins.

endogenous label

In this experiment recovery of ^{13}C was measured for intraperitoneally injected [1-^{13}C]-leucine.

◇ for the BAA diet

In experiments within BAA-diet group, the ^{13}C expiration from IP injected tracer was measured without a meal (post absorptive) and in the presence of BAA meal. The ^{13}C expiration is comparable in both situations (a sharp peak after 30 min). When a meal was given, the response and the cumulative [^{13}C] recovery from endogenous [l-^{13}C]-leucine tended to be higher compared to the situation in the absence of a meal. This means that there was certainly no decrease in catabolism of endogenous AAs in presence of a BAA meal. In other words, there was no decrease to compensate for the increase in catabolism of exogenous AAs.

◇ for the FAA diet

Three nutritional situations were breath tested with IP injected label in the animals fed the FAA diet: 1. no meal (control-post absorptive situation); 2. FAA meal; 3. FAA meal without leucine.

Significantly lower catabolic losses of leucine from endogenous AA pool occurred at 30 min through 120 min when FAA-Leu meal was given compared to a complete free amino acid (FAA) meal (figure 3). Total cumulative label recovery after 7.5 h was also lower for the FAA-Leu group.

From those results, it can be concluded that amino acids from a meal influence the catabolism of AAs from the endogenous AA pool. The influence was more pronounced with free amino acids in the meal than with egg white bound AAs. In case of a complete FAA meal, the catabolism of leucine of the endogenous AA pool was increased. On the contrary in the case of a FAA-Leu meal, leucine of the endogenous AA pool was spared. In the case of a complete meal the amount of AAs entering the endogenous AA pool may exceed the capacity of AA synthesis. Those AAs remain in the body pool and they will stimulate their own catabolism. In the case of leucine deficient meal the losses of leucine from the endogenous AA pool are lower. Due to

leucine deficiency of the AAs entering the body pool, leucine from the endogenous AA pool will be used for protein synthesis. This could explain the lower catabolism.

Absorption of dietary AAs into the endogenous AA pool

In this study, it was investigated whether, and for how long, dietary and endogenous AAs are handled independently by the body or that they mix up completely prior to metabolism. It is possible that dietary AAs are metabolised in the intestinal cells whereas endogenous AAs are metabolized in the AA pool. In this situation, there would be two separate pools.

It is not taken for granted that all dietary amino acids absorbed by the small intestinal mucosa enter the portal circulation and become available to extraintestinal tissues (Windmueller et al., 1982). Experiments based on the direct measurement of the net portal appearance of amino acids in pigs suggest that considerably less than 100% of the dietary amino acids appear as free amino acids in the portal blood (Ebner et al. 1994, Rerat et al. 1988 and 1992). It is claimed for 4 week old female pigs that roughly one third of dietary intake of essential AAs is consumed in first-pass metabolism by the intestine with greater catabolism than AAs incorporation into mucosal protein (Stoll et al., 1998)

In the present study when a complete meal was given, the [^{13}C] recovery from IP injected [1-^{13}C]-leucine tended to increase compared to the control curve. This increase of catabolism suggests a response to an influx of AAs into the endogenous AA pool caused by the dietary AAs. Therefore, we assume that dietary leucine starts to mix up with the endogenous AA pool rapidly after ingestion. Quantitative data cannot be derived from these measurements.

The increase in catabolism of leucine from the endogenous AA pool after the meals containing about 53mg of leucine was only minor. This suggests that the endogenous flux of leucine is considerably higher than the influx due to the meal.

We conclude that only part of the absorbed dietary AAs enters the endogenous AA pool immediately to mix up with the endogenous AAs after absorption. A large

part of the dietary amino acids does not even reach the endogenous AA pool since they are utilised by the intestinal cells. Nevertheless, the response of endogenous catabolism upon a meal intake was seen as either an increase or decrease depending on the dietary supply.

The lack of differences between the control curves of the [^{13}C] expiration from IP injected [1-^{13}C]-leucine for the BAA (figure 2) and FAA-diet (figure 3) is worth mentioning. The injected [1-^{13}C]-leucine represents the leucine in the endogenous AA pool of the body in post absorptive state. The two diet groups did not significantly differ in ^{13}C expiration when no meal was provided. This suggests no influence of the FAA diet on post absorptive protein metabolism.

The short-term catabolism of leucine of the endogenous AA pool in the post-prandial phase was influenced by the dietary form and pattern of AA in the meal. In the case of FAA meal without leucine catabolism of leucine from endogenous AA pool was decreased suggesting that endogenous leucine was used for net protein synthesis. In the case of a non-deficient meal, there is an extra loss of endogenous AAs beyond the extra loss of exogenous AAs. Therefore free amino acid replacement of protein bound amino acid does not seem to be without physiological consequences. Supplementation of FAAs in clinical nutrition or animal nutrition to meet requirements can induce extra catabolism on exogenous but also on endogenous side. It is to be expected that AA supplementation in food is most profitable when catabolism of endogenous AAs is not affected.

CONCLUSIONS

1) Short-term catabolism of dietary free leucine considerably exceeds catabolism of egg white bound leucine.
2) Short-term catabolism of endogenous amino acids is modulated by exogenous amino acid supply.
3) Short-term catabolism after a meal is not restricted to dietary amino acids itself but also holds to some extent for the endogenous amino acids present in the AA pool of the body. At least a part of the dietary leucine mixes up with the endogenous AAs immediately after absorption since dietary amino acids influenced the catabolism of endogenous AAs in this experiment very quickly.
4) Short-term catabolism of endogenous AA increased or decreased depending on the adequacy of the dietary supply.

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Chapter 2

A 1h time interval for free methionine supplementation reduces post prandial amino acid oxidative losses of methionine deficient dietary protein.

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ABSTRACT

The recovery of a label expired as [$^{13}CO_2$] was measured in the exhaled air of rats after ingestion of egg white bound [1-^{13}C]-leucine or free [1-^{13}C]-methionine. Rats were fed a methionine deficient meal and after various time intervals a free methionine supplement mixed with non-protein diet components. The rats were fed 3 meals a day. The 1st (main) meal (50% deficient in methionine) was fed at 9:00h. The 2nd (supplementary) meal was given 0h, 1h, 2h and 4h after meal 1. At 16:30h a 3rd meal (full) was given. [$^{13}CO_2$]-breath tests were performed on 5th and the 26th day on the experimental diet. The cumulative label recovery from egg white bound [1-^{13}C]-leucine (given with meal 1) was highest for the 4h and lowest for 1h interval. Recoveries of ^{13}C from free [1-^{13}C]-methionine (given with meal 2) were highest for the intervals that showed lowest oxidative losses of [1-^{13}C]-leucine. For [1-^{13}C]-methionine recovery values were much higher than for [1-^{13}C]-leucine ($p<0.05$), probably due to conversion to cysteine. After 26 days on the diet the [$^{13}CO_2$]-breath test characteristics were similar to those on the 5th day. Rats of all interval groups gained weight at the same rate. These results illustrate time lag in metabolic availability of free and protein bound AAs[1]. As a consequence, a proper time interval between protein bound and free AAs can reduce post prandial AAs' oxidative losses. In this case, free methionine supplementation given 1h after the deficient meal gave lowest post prandial oxidative losses, allowing better utilization.

Keywords: [1-^{13}C] methionine, [1-^{13}C] leucine, amino acid supplementation

[1] Abbreviations used: AA(s), amino acid(s); AT%, atom percent

INTRODUCTION

Meal feeding is associated with gain during and after a meal (post prandial) and with mobilization of nutrients between meals (post absorptive). In the case of protein, these two processes are called "diurnal protein cycling" (Millward, 1998). Levels of free AAs in the blood are kept low because of their toxicity. Therefore, AAs of dietary origin are transformed to body proteins and stored as such (Millward, 1992). When the appearance rate of dietary AAs in blood exceeds the protein synthesis capacity to store them, the surplus is channelled into energy metabolism. This energetic utilization is initiated mostly by decarboxylation. At least in rats a substantial part of the dietary amino acids (ca. 20%) may be lost from the body by early oxidation during the post prandial phase of a meal (Weijs et al., 1993). It is expected that post prandial oxidative losses of dietary AAs can be modulated by dietary habits or by specific feeding strategies. Both have an impact on the amino acid appearance rate in the blood. At restricted protein levels, poor quality diets have to be supplemented with free AAs to meet individual AAs requirements. Various studies have indicated that dietary AAs in free form are sometimes less well utilized than protein-bound AAs (Batterham and Bayley, 1989; Deschepper and De Groote, 1995; Daenzer et al., 2001). Metges et al. (2000) proved that a time-lag between oxidation of free and bound AAs may occur. Therefore, it is important to examine the optimal way of free AAs' supplementation. In a physically active person who ingests AAs supplements not only the exact composition and amount of an amino acid supplement is important, but also the timing of ingestion of the supplement in relation to the exercise has been considered (Rasmussen et al., 2000; Wolfe, 2000; Tipton et al., 2001). We hypothesize that many nutritional conditions, such as the AAs pattern of a feed protein, the amount of protein per feeding, the feeding frequency, the presence of synthetic amino acids, the time of free AA supplementation in relation to the deficient meal, size of the meals, etc. will influence the appearance rate of AAs in blood. All these factors will affect the level of post prandial AA oxidation. To our knowledge the optimal time interval for free amino acid supplementation of

deficient protein is not considered as a factor for improving amino acid utilization. Therefore, in the present experiment we used a free methionine supplement given at different time intervals after a methionine deficient meal.

Hence, the purpose of this model study was to investigate the importance of the timing of free methionine supplement after methionine deficient protein upon post prandial loss of dietary amino acids in rats. The [$^{13}CO_2$]-breath test technique was performed to study oxidative losses of a label. We used egg white bound [1-^{13}C]-leucine (always in the 1st meal) and free [1-^{13}C]-methionine (always in the 2nd meal) as oral substrates.

MATERIALS AND METHODS

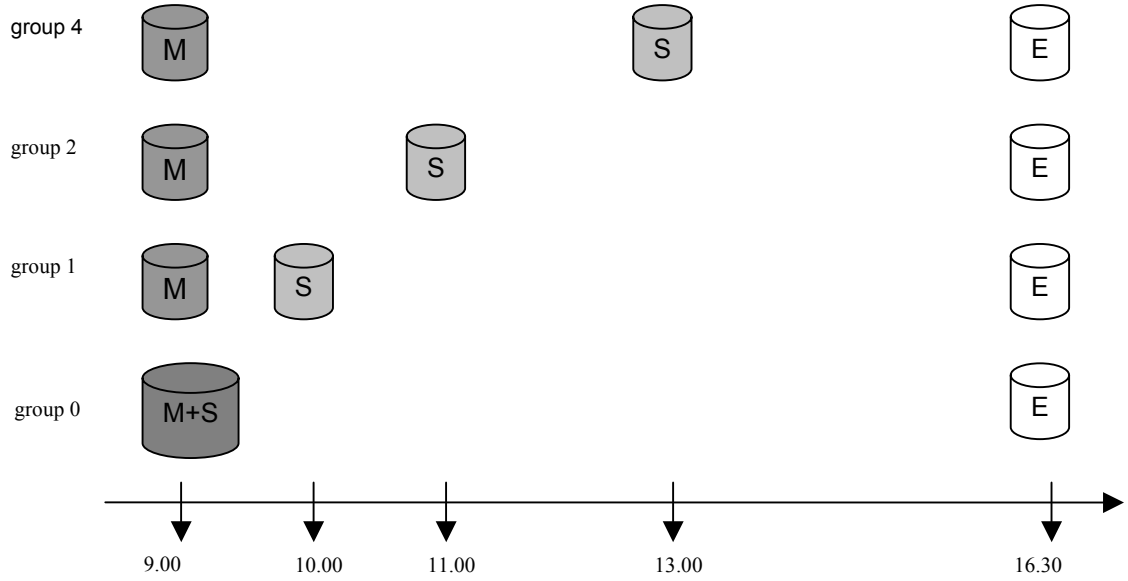
The experimental aim was to investigate the influence of time interval between methionine deficient protein meal and its free methionine supplement by post prandial oxidative losses of label from egg white bound [1-^{13}C]-leucine and free [1-^{13}C]-methionine. [$^{13}CO_2$]-breath test measurements (recovery of ^{13}C in expired CO_2) were performed on 5th and 26th day after the start of the experimental treatment to study metabolic adaptation. The protocol for the experiment was approved by the Ethical Committee of Wageningen University.

Animals

Forty eight male WU-Wistar rats, supplied by Harlan company (The Netherlands), with an initial weight of approximately 60g, 3-4 week old, were randomly allotted to one of the four "interval" groups (0, 1, 2 and 4 hours). The rats were caged individually and housed in a room controlled temperature at 22 ^{o}C and 70% humidity. The rats were conditioned on a light schedule with 16 hours of light and 8 hours dark (9:00h to 17:00h). Tap water was available 'ad libitum'. At the end of the experiment the animals were killed with an O_2/CO_2 gas mixture (ratio 1:2).

Feeding strategy

In scheme 1 the feeding strategy of the animals is shown.



Scheme 1. Feeding schedule.

M - main meal (with protein 50% deficient in methionine), on measurements day with egg white bound [1-^{13}C]-leucine; **S** - supplementary meal (with free methionine), on measurement day with free [1-^{13}C]-methionine; **E** - evening meal (with egg white bound protein)

During a two-week conditioning period the animals were conditioned to receive 3 meals a day for 30 minutes each. The meals contained 45, 15 and 40% of daily energy supply. The 1st and the 3rd meal were always given to all groups at the same time at the beginning (09:00h) and at the end of the dark period (16:30h), respectively. There were four experimental treatments related to the time interval for the 2nd supplementary meal after meal 1. Groups 0, 1, 2 and 4 were given the supplement with an interval of 0, 1, 2 and 4 hours at 9:00, 10:00, 11:00 and 13:00 hours, respectively. The 0h interval was chosen because it is used in practice simply by mixing the supplement with the deficient protein meal. The 4 h interval was included because we expected it as probably being too late to act as an effective AA supplement for the deficient meal. The 1h or 2h interval was expected to give a better result than the 0h and 4 h treatments. After conditioning to 3 meals a day for two weeks, the schedule was

continued with the experimental diets. Total daily feed intake for all animals was determined by the animal, which ate the lowest amount. The intake was gradually increased until the first measurements (5^{th} day on experimental feed). Thereafter the food intake was kept at 11.2g a day until the end of the experiment.

Diets

During conditioning to the daily meal pattern the animals received the same full diet with all 3 meals (diet 3). After 2 weeks of conditioning the compositions of meal 1 and 2 (morning feeding) were changed. The morning feeding as a whole represented a full diet. However the protein fraction (egg white + vegetable protein) was 50% deficient in methionine. The deficiency was compensated by free methionine. The protein derived and free amino acids of the diet were provided with meal 1 and 2, respectively. The methionine was supplemented in free form in the second protein free meal along with other free AAs in minor amounts to meet the total requirements. Ingredients of the supplementary diet were easily digestible with glucose as the major energy source and some cellulose for structure. Meal 3 remained the same as during the conditioning time. The composition of the meals is shown in table 1.

Table 1. Diet composition[2]

Feed ingredients (g)	**Morning meal (60% of daily intake)**			**Evening meal (40% of daily intake)**
	Total[3]	**1**	**2**	**3**
		Met deficient protein diet	diet with free Met supplement	**Diet 3**
	100%	75%	25%	100%
Chicken egg white powder	50	50		132
Soyabean meal	50	50		-
Peas	150	150		-
Sugar	50		50	-
Wheat	250	250	-	-
Dextrose	-		-	195
Wheat starch	285	225	60	475
Cellulose	50	10	40	75
Vegetable oil	45	15	30	50
Vit. Premix rat	12	-	12	10
Min. Premix rat	10	-	10	12
CaCO3	18	-	18	19
MgO	1	-	1	1.5
NaH2PO4.2H2O	4	-	4	6.5
KCL	0	-		0.5
KH2PO4	10	-	10	14
KHCO3	4	-	4	10
NaCl	1	-	1	-
L-isoleucine	0,2	-	0,2	-
L-leucine	0,9	-	0,9	-
L-lysine HCl	2,5	-	2,5	-
DL-methionine	4,7	-	4,7	-
L-threonine	1	-	1	-
L-tryptofan	0,2	-	0,2	-
Total	1000	750	250	1000

[2] tabulated requirement for Met+Cys for growing rats - 9.7g/kg (NRC)
[3] calculated Met+Cys content in the total morning meal 9.7g/kg

MEASUREMENTS

Body Weight development

To study body weight development all animals were weighed daily just before the morning meal at 9:00 o'clock.

[$^{13}CO_2$] - Breath test

The oxidative losses of amino acids during post prandial phase of the meal were monitored by [$^{13}CO_2$]-breath test measurements. A [$^{13}CO_2$]-breath test is a simple and non-invasive method and is known as a helpful tool to study various metabolic processes (Schreurs et al., 1992; Raguso et al., 1999; Reeds et al., 1999). On 5th and 26th day of the experiment [$^{13}CO_2$]-breath tests were conducted to measure the oxidative loss of labeled dietary components. The amount of ^{13}C recovered, in the breath after a meal, reflects oxidative loss of dietary amino acids. The non-oxidized AAs remain available for other metabolic processes. This implies that a situation with the lowest (cumulative) recovery of label from protein bound [1-^{13}C]-leucine is assumed to represent the optimal nutritional situation. It gives the least losses and allows the highest post prandial protein synthesis. On the day of measurements for half of the animals (n=24) the egg white in meal 1 was replaced by enriched (ca. 1.48 At%) [1-^{13}C]-leucine egg white protein (375 mg dry matter). The egg white was produced according to Evenepoel et al. (1997). For the other half of the animals 6.8 mg of free methionine in the second meal was replaced by [1-^{13}C] methionine (chemical purity > 99%, isotopic enrichment > 99%, Mass Trace, Woburn, USA). To collect expired air rats were placed individually in airtight macrolon cages (20 cm x 16 cm x 14 cm) bedded with sawdust with ad libitum access to water. Each half an hour a 50 ml air sample was taken from the cage with syringe fixed on a lid. After each sampling period of 30 minutes rats were placed in a clean cage with fresh air. Air sampling started half an hour prior to feeding the label to get a blank value for the [^{13}C] At% enrichment of expired air of the animal. Thereafter, animals received their labeled experimental meal.

The non-labeled meals were given at usual times. In total 11 breath samples per animal were collected during 5.5 hours of measurements.

The collected samples were analyzed by a Finnigan Delta C IRMS (Isotope Ratio Mass Spectrometer, Finnigan, Bremen, Germany) at the WIAS-IRMS facility at the Department of Animal Sciences. The samples were analysed for [^{13}C]-enrichment of CO_2 (At%). Results are expressed as rate of recovery per hour and as cumulative recovery over the entire 5 hour collection period.

Total CO_2-production

We measured the $^{13}C/^{12}C$ ratio in the air. Total CO_2-production values were needed to calculate recovery of ^{13}C. Values for the CO_2-production were derived from total CO_2-production measurements, which were performed in 16 animals (n=4 for each interval group) with similar body weight and food intake. During the CO_2-production measurements cages were continuously ventilated with fresh air. The flow of outgoing air was measured (ca 0.5 l/min) and air was dried with SPOCA (dried sponge with $CaCl_2$, which absorbs water), after which air was analyzed in a calibrated CO_2-analyser (Hartman & Braun, Uras 3G). CO_2-production was calculated as the mean of the 30 minutes periods. It was noticed that the results were rather constant in time and did not differ among treatments. Therefore, the mean value of CO_2-production was used as an estimate for all the animals for the whole measurement period (0.13 lCO_2/30min on the 5^{th} day and 0.17 lCO_2/30min on the 26^{th} day of the experiment).

The MANOVA variance analysis and post hock LSD test (Statgraphics Plus, version 4.1) were used to observe statistically significant differences.

RESULTS

Body weight development

In order to check whether different supplementation strategies had any influence on body weight development, rats were weighed daily just before the morning meal at 9.00 o'clock. Rats on different treatments gained weight at the same rate. No significant differences in weight between groups were observed on the 1st (104±3g) and 26th day (181±6g) of the experimental diet. Average weight gain till the 5th day on experimental diets (1st breath test measurements) for all the animals was 9±2g. Till the 26th day of the experimental treatment (2nd breath test measurements) mean weight gain was 79±5g.

[$^{13}CO_2$]-breath test

The rate of ^{13}C recovery from orally ingested labeled substrate was expressed as a percentage of the ingested dose per hour. It was monitored as $^{13}CO_2$ in the breath for 5 hours after meal 1 for recoveries of label from egg white bound [1-^{13}C]-leucine and after meal 2 for recoveries of label from free [1-^{13}C]-methionine, in separate experiments.

The curve of group 4 (figure 1) is described first since this curve can be considered as a blank response to meal 1 without supplementation (0-4 hours). The rate of label recovery from egg white bound [1-^{13}C]-leucine increases to a maximal value of 4.0% of ^{13}C intake per hour after 150 minutes. Thereafter, the recovery declines and has a value of 2.1% per hour at the end of measurements (300 min).

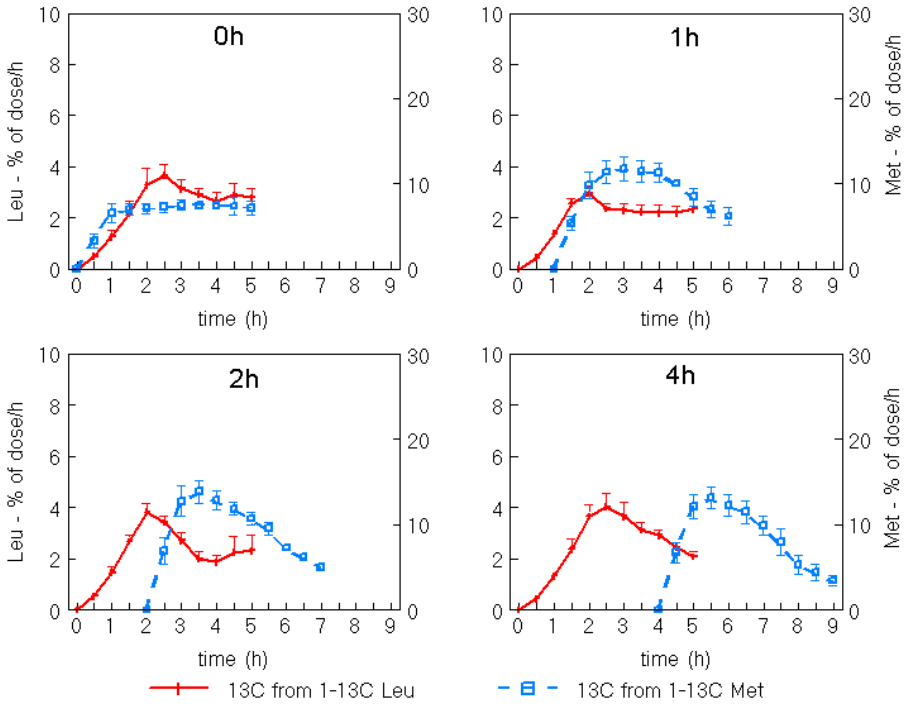


Figure 1. Rate of ^{13}C recovery (% of dose/hour ±SD) derived from egg white bound [1-^{13}C]-leucine (n=6 for each interval group) and free [1-^{13}C]-methionine (n=6 for each interval group) during 1st breath test's measurements (5th day on experimental diet).

0, 1, 2 and 4h groups received main deficient protein meal followed by the free Met supplement: together with a main meal, after 1, 2 or 4 hours, respectively.

When the supplementary meal is given 2 hs after the first protein deficient meal, the label recovery starts to decline after 120 minutes from a level of 3.8% of dose/h until 1.9% of dose/h at 240 min. In this treatment the plateau was reached later and at the level of ca. 2.3% of dose/h after 300 minutes.

In group 1 label recovery starts to decrease after 120 min. but the level reached is only 2.9%. Thereafter the curve declines to a value of 2.3% and stays at this level from 150 min. on until the end of measurement (300 min).

The recovery curve of group 0 that received the deficient meal mixed with the supplement increases until 150 min to a level of 3.7%. Then the curve

slowly declines until 240th minute and keeps plateau level of ca. 2.8% till 300 min.

Results of the 2nd breath test session after 26 days on experimental feeding are shown in figure 2. After 26 days the characteristic patterns for the recovery of label from the protein fraction were similar to those from the measurements on the 5th day. But the cumulative values were significantly higher ($p<0,0001$). The curve of group 0 is less peak shaped compared to day 5. It shows a higher plateau value after 120 minute at the level of ca. 3.5% at day 26.

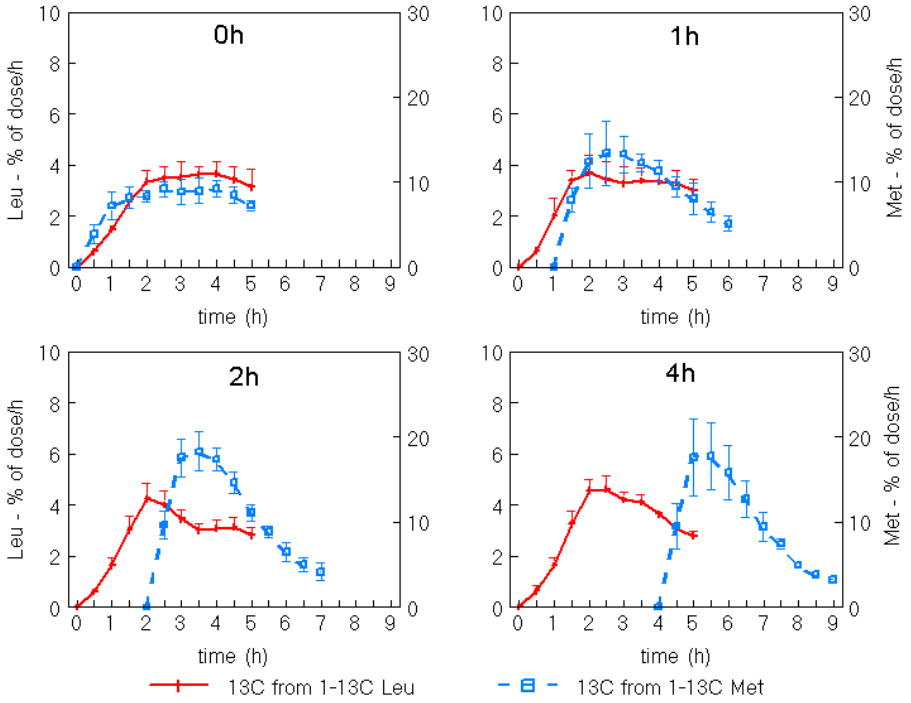


Figure 2. Rate of ^{13}C recovery (% of dose/hour ±SD) derived from egg white bound [1-^{13}C]-leucine (n=6 for each interval group) and free [1-^{13}C]-methionine (n=6 for each interval group) during 2nd breath test's measurements (26th day on experimental diet).

0, 1, 2 and 4h groups received main deficient protein meal followed by the free Met supplement: together with a main meal, after 1, 2 or 4 hours, respectively.

The values for the cumulative recoveries of label from egg white bound [1-^{13}C]-leucine are presented in table 2. The cumulative recovery of the label after 5 days on experimental feeding for the animals in the 1h group was significantly lower than in groups 0h and 4h.

Table 2: The 5h cumulative recovery of label (% of dose ±SD) of ^{13}C derived from egg white bound [1-^{13}C]-leucine and free [1-^{13}C]-methionine in two sessions of breath test (BT) in groups with different time interval (0, 1, 2 and 4 (n=6)) between meal with methionine deficient protein and its supplement. Significant differences between groups (p<0.05) are indicated by different letters; small letters-1st breath test, capital letters-2nd breath tests;

group	**[1-^{13}C]-leucine**		**[1-^{13}C]-methionine**	
	5th day (1st BT)	26th day (2nd BT)	5th day (1st BT)	26th day (2nd BT)
0	12.6±1.4^{bc}	14.4±1.4^{A}	34.1±2.2^{a}	39.9±3.0^{A}
1	10.5±0.5^{a}	14.8±1.5^{A}	46.4±3.4^{bc}	49.8±3.1^{B}
2	11.6±1.0^{ab}	14.5±1.3^{A}	48.5±3.5^{c}	56.6±2.6^{C}
4	13.0±1.0^{c}	16.3±1.0^{B}	43.4±4.6^{b}	51.1±8.5^{B}

MANOVA

Influence	**[1-^{13}C]-leucine**	**[1-^{13}C]-methionine**
t - time interval	p<0,0009	p<0,0001
a – 3 weeks of adaptation	p<0,0001	p<0,0001
interaction t×a	p<0,1031	p<0,5293

After 26 days on the treatment the total recoveries from [1-^{13}C]-leucine between groups 0, 1 and 2 were similar. The values for animals in group 4 remained significantly higher compared to the other groups.

The graphs of the rate of label recovery from free [1-^{13}C]-methionine as an indicator of oxidative losses of supplemented Met (Figure 1) show that groups 2 and 4 have similar curves. A maximum of 13.9 and 13.1% per hour is reached

90 minutes after ingestion of the tracer with the supplement in groups 2 and 4 respectively. Group 0 (animals which received a mixture of deficient meal and methionine supplement) reaches a nearly constant level of ca. 7% after 60 minutes. The situation for group 1 is intermediate. The maximum recovery of 11.5% is reached after 150 minutes. Later it decreases to the level of 6.2% of dose/h at the end of measurements after 300 minutes.

The second breath test's curves for label recovery from free [1-^{13}C]-methionine on day 26 (Figure 2) show the same patterns as in the first breath test on day 5. The cumulative recovery values are somewhat higher.

The total cumulative recovery of label from free [1-^{13}C]-methionine after 5 hours of measurements is presented in Table 2. On the 5th day of the experimental diets group 0 shows a significantly lower recovery of label compared to other groups ($p<0.0001$). The highest values were obtained for animals in group 2. They were not significantly higher than for animals in group 1. The cumulative recovery of label for animals in group 1 was significantly higher than in group 4. After 26 days of the experiment, group 0 had significantly lower cumulative label recovery from free [1-^{13}C]-methionine than other groups. Group 2 showed significantly higher values than the remaining groups. Animals treated with 1 and 4 hours intervals had intermediate cumulative label recovery and did not differ between each other. The recovery of label from [1-^{13}C]-methionine is remarkably higher than that from egg white bound [1-^{13}C]-leucine.

DISCUSSION

The aim of the study was to derive an optimum time interval between a meal with methionine deficient protein and a second meal with the compensatory supplement of free methionine. The post prandial oxidative losses of egg white bound [1-^{13}C]-leucine and free [1-^{13}C]-methionine and body weight development of growing rats were measured. On the 5th day of experimental feeding the first breath test with labeled egg white bound [1-^{13}C]-leucine was performed. This test allows the estimation of the proportion of amino acids that are irreversibly lost from protein metabolism by decarboxylation (Reeds and Davis, 1999). The non-oxidized AAs remain available for protein synthesis. The treatments with the lowest total cumulative recovery of label from protein bound [1-^{13}C]-leucine reflects the lowest losses. The second breath test was performed after 3 weeks on the nutritional treatment. This was done to study adaptation to feeding strategy. We arbitrarily assumed that after 5 days animals are still not fully adapted to diet change on day 0.

Figure 1 shows that the label recovery from egg white bound [1-^{13}C]-leucine is already detectable 30 minutes after consumption of a meal. The pattern of recovery for first 90 minutes was similar for all interval groups. Differences between groups occurred from 120th minute onwards.

When both deficient protein and supplement are mixed together (group 0), free supplementary amino acids are metabolized at an earlier stage than AAs bound in protein. It can be argued that when both free and protein bound AAs are given at different times the oxidation of AAs will be lower than when protein and free AAs are given at the same time. Indeed post prandial cumulative recovery of label from [1-^{13}C]-leucine on treatment without time interval (group 0) is higher than with 1 hour interval. Group 0 was similar to the 4 hour interval group. This means that both the 0 hour and 4h interval cause an avoidable loss of leucine. Therefore with a 0 hour and a 4 hour interval there are lower availabilities of AAs for protein synthesis than in case of 1 hour.

Rate of label recovery from [1-^{13}C]-methionine (Fig. 1 and 2) in the 0 group shows a faster increase (during the 1st hour) than the recovery from [1-^{13}C] leucine, which is bound in the protein. This indicates that when both the free methionine and the protein bound AAs are given at the same time free methionine reaches the body pool faster than amino acids of protein origin. Label recovery of this treatment reflects a quick absorption of the free methionine after the meal. Thus AAs from protein are absorbed at a later time. So the gastrointestinal tract separates absorption of free and bound AAs ingested with the same meal. This explains a higher label recovery from egg white bound [1-^{13}C]-leucine in case of one big mixed meal (protein + free methionine). The lowest post prandial losses of protein occurred when the time interval between the two meals was 1 hour. So this time period potentially may be the most beneficial for protein synthesis. It has to be stressed that the optimal time interval may depend on both the nature of the dietary protein and the supplemented AA. In addition it may differ between species.

When post prandial nutritional situation allowed synchronization of dietary AAs appearance rate (groups 1 and 2) then the cumulative recovery of label from bound [1-^{13}C] leucine was lowest. Interestingly, in the case of lower label recovery from egg white bound [1-^{13}C]-leucine there was higher label recovery from free [1-^{13}C] methionine. This can be explained as follows. Methionine, in addition to its direct role in protein synthesis, serves as an S-donor in cysteine synthesis (Finkelstein, 1998). This transsulfuration reaction also leads to release of $^{13}CO_2$ from [1-^{13}C]-methionine in addition to its direct oxidation. Therefore, the oxidative losses of labeled methionine may not be considered completely as loss but, at least in part, as a contribution to cysteine synthesis. An increased requirement for cysteine for protein synthesis might therefore increase recovery of ^{13}C from [1-^{13}C]-methionine as measured by ^{13}C (Raguso et al., 1999).

Based on the cumulative recovery values it can be concluded that diets with methionine deficient protein and free AAs given simultaneously do not have

the highest utilization. The lowest oxidative losses were obtained when the methionine was given one hour after the methionine deficient meal. With a 4-hour interval the lowest efficiency was found. So we can conclude that the optimum time interval will depend on the rate of dietary protein hydrolysis and absorption of AAs from dietary protein.

The second aim of the study was to examine whether adaptation to the same feeding pattern occurs. It was done by comparing 5^{th} and 26^{th} day on the feeding procedure.

Results show that after 3-week adaptation period post prandial cumulative label recovery from both [1-^{13}C]-leucine and [1-^{13}C]-methionine significantly increased. The total level of label recovery after 26 days was about 1.3 times higher than after 5 days of the treatment. It should be noted that rats were depositing more protein on day 5^{th} than on day 26^{th} and also animals were somewhat heavier on day 26. Both will increase oxidation.

At the beginning of nutritional treatment label recovery from [1-^{13}C]-leucine with methionine at 0h interval was higher than in the group 1. After 3 weeks this difference disappeared. Only the post prandial label recovery from [1-^{13}C]-leucine remained at a highest level in group 4 where both meals were 4 hours apart.

The study indicated that various time intervals between the main methionine deficient protein meal and the supplement caused differences in post prandial losses of dietary amino acids. These losses, however, did not influence weight gain. This suggests that overall availability of dietary amino acids was not limiting for weight gain.

CONCLUSIONS

1) The best utilization of methionine deficient protein does NOT occur when methionine supplement is given simultaneously with methionine deficient meal.
2) A time interval between methionine deficient protein meal and its supplement in free form lowers the post prandial oxidative losses of protein derived AAs (leucine).
3) In the present case free methionine supplementation given one hour after the main methionine deficient meal tends to allow better post prandial protein utilization. This can be concluded from the lowest label recovery from egg white bound [1-^{13}C]-leucine at this time difference.
4) Optimal post prandial nutritional situation for protein bound leucine coincides with higher label recovery from [1-^{13}C]-methionine, probably due to conversion to cysteine.
5) After 3 weeks, higher recoveries of label from both [1-^{13}C]-leucine and [1-^{13}C]-methionine were observed probably due to a larger body mass and lower growth rate of animals.

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Chapter 3

Influence of free methionine supplementation interval on post prandial amino acid catabolism and body composition at different levels of protein intake.

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ABSTRACT

In the present study it was examined in growing rats whether dietary protein levels of 5, 7.5, 10 and 13.8% affected the post prandial metabolism of free amino acids when these were given at the same time as the diet or with an 1 hr interval. The amino acid pattern of the protein fraction was 50% deficient in methionine but supplemented with free methionine within the feeding strategy. In the morning and in the afternoon rats were fed the protein fraction and the free methionine supplement either as a mixture (0h interval) or with a time interval of 1h. The impact of the supplementation strategy on post prandial amino acid oxidation was studied after the rats had been on the diet for 5 days. Effects on weight gain and on body and liver composition were measured after 32 days.

Weight gain and body composition were clearly influenced by the protein level of the diet. Supplementation with methionine at a time interval of 1h improved weight gain when the 5% protein diet was given. This beneficial effect was reduced when the protein level in the diet was higher than 5%. In case the animals were fed the diet with the high dietary protein level (13.8%), the content of protein was higher while the content of fat in the dry matter (DM) was lower compared to animals on the 5% protein diet. Fatty livers were observed in animals fed the 5% protein diets. The presence of a time interval between the main diet and the free methionine supplementation influenced weight gain and body composition without having a significant impact on the composition of the livers.

Keywords: [1-^{13}C] methionine, [1-^{13}C] leucine, amino acid supplementation

INTRODUCTION

Poor quality diets with a deficient amino acid pattern are normally supplemented with free crystalline amino acids. A supply of extra protein can have the same beneficial effect. Adequate supplementation is critical when the total nitrogen intake with the diet should be marginal. However, free amino acid supplementation is not left without physiological consequences. A higher and more rapid increase followed by more rapid decline in individual plasma amino acid concentrations has been observed after ingestion of free L-amino acids or in combination with whole protein, compared to ingestion of an equivalent amount of amino acids in the form of dietary protein (Gropper et al. 1991 and 1993).

The risk of decarboxylation could make free amino acid supplementation less effective resulting in a lower protein gain than expected.

The classical way of improving poor quality protein diets is by mixing the diet with a supplement of the missing amino acid(s) in free crystalline form (single supplementation). In chapter 2, different time intervals for intake of methionine supplementation together with or after the morning methionine deficient meal have been studied. The effects on the post prandial oxidation of dietary proteins and the supplemented methionine were subsequently measured. Introduction of a 1 h interval between the amino acid deficient meal and its free amino acid supplement reduced the post prandial oxidative losses of egg white bound [1-^{13}C]-leucine from the deficient meal. These differences in post prandial oxidation were not reflected in body weight changes among groups with different time intervals of supplementation. The present experiment aimed to investigate whether post prandial oxidation of leucine or methionine was affected by an 1 h time interval between meals of different protein content and supplement with free methionine. In addition, the possible consequences of this strategy for weight gain and body composition of rats were studied.

MATERIALS AND METHODS

Animals

Forty-eight male Wistar rats (119 ± 8g at the start of the experiment) were allotted to one of the four dietary groups (diets consisted of 5, 7.5, 10 or 13.8% protein). Each protein group was further divided in two sub-groups that received the free methionine supplement with a time interval of 0 or 1 hour after the main meal. The rats were caged individually at a temperature of 22 ^{o}C and 70% humidity. They were conditioned to a light schedule with 15 hours of light and 9 hours dark (9 am to 6 pm). Tap water was available 'ad libitum'. The protocol for the experiment was approved by the Ethical Committee of Wageningen University.

Feeding regimen

Animals were fed twice a day in the morning (09:00) and in the afternoon (16:30). During first two weeks the 0h interval groups were conditioned to receive 2 and the 1h interval groups 2 x 2 meals a day. Eating was allowed for 30 min/ meal. The animals received 60% of total daily intake in the morning and 40% of the intake in the afternoon. For the 1h interval group the total energy ratio of the protein and free amino acid supplemented sub meal was 4 : 1. For the 0h interval group the protein and free amino acid supplement were mixed and supplied at 09.00 and 16.30 hour. In case of the 1h interval group the protein containing meals were also provided at 09:00 and 16:30 and the free amino acid supplemented sub-meal at 10.00 and 17.30 hour.

To avoid variation in feed intake during the experimental period the food supply was determined by the least eating animal. When all the animals ate the feed completely, the next day there was slight increase (0.1-0.2 g a day) in the size of the feed supply. If there were some leftovers the feed supply was reduced or kept at the same size as the previous day. It was observed that if there were any leftovers they were in the group fed the 5% protein diet. As a

consequence of this approach, the amount eaten by one rat during the experimental period increased from 10 g to 12 g a day.

Diets

During the conditioning to housing situation and feeding strategy, the animals received commercial rat chow (Teklad Global Rodent Diet; Harlan, Horst, The Netherlands). The experimental diets were prepared by Diet Research Services (Wijk bij Duurstede, The Netherlands). The experimental diets contained 5, 7.5, 10 or 13.8% crude protein. The amino acid pattern of the protein fraction in the diets was calculated to be 50% deficient in methionine with respect to the official recommendations (NRC, 1995). The composition of the diets is shown in Table 1. Part of the protein fraction consisted of egg white protein. This protein source was chosen with the objective to replace it by intrinsically labeled egg white protein during the [$^{13}CO_2$] breath test measurements. The relative size of the meals is described under feeding regime.

Table 1. Experimental diets

Protein level →	13.8 %			10.0 %			7.5 %			5.0 %		
Meal →	Total	sub 1	sub 2	Total	sub 1	sub 2	Total	sub 1	sub 2	Total	sub 1	sub 2
Ingredients (in g) ↓	100 %	80 %	20 %	100 %	80 %	20 %	100 %	80 %	20 %	100 %	80 %	20 %
Peas	430.0	430.0		310.5	310.5		230.5	230.5		150.5	150.5	
Chicken egg white	50.0	50.0		36.1	36.1		26.8	26.8		17.5	17.5	
DL-methionine	5.0		5.0	3.6		3.6	2.7		2.7	1.8		1.8
Dextrose	50.0		50.0	50.0		50.0	50.0		50.0	50.0		50.0
Wheat starch	338.2	240.6	97.6	440.7	370.6	70.2	509.6	441.0	68.6	578.6	511.4	67.1
Vegetable oil	35.0	30.0	5.0	36.5	20.0	16.5	37.5	20.0	17.5	38.5	20.0	18.5
Cellulose	30.0		30.0	56.5	10.0	46.5	74.5	27.0	47.5	92.5	44.0	48.5
Vit. premix	12.0	9.6	2.4	12.0	9.6	2.4	12.0	9.6	2.4	12.0	9.6	2.4
Min. premix	10.0	8.0	2.0	10.0	8.0	2.0	10.0	8.0	2.0	10.0	8.0	2.0
Total (g)	1,000.0	800.0	200.0	1,000.0	800.0	200.0	1,000.0	800.0	200.0	1,000.0	800.0	200.0
Calculated values (g/kg)												
Crude protein	141			102			77			51		
Crude fat	40			40			40			40		
Crude cell material	53			53			85			98		
Crude ash	45			44			43			42		
ME pl (kcal/kg)	3,208	2,569	643	3,208	2,569	643	3,208	2,569	643	3,209	2,569	643
Leucine	10.4			7.5			5.6			3.7		
Methionine	7.6	2.6	5.0	5.4	1.8	3.6	4.0	1.3	2.7	2.6	0.9	1.8
Methionine+Cys	**9.8**	**4.8**	**5.0**	**7.0**	**3.4**	**3.6**	**5.2**	**2.5**	**2.7**	**3.4**	**1.7**	**1.8**

Abbreviation: sub = submeal

Measurements

Body weight development

All animals were weighed daily prior to the main morning meal at 09:00 hour.

[$^{13}CO_2$] - Breath test- day 5

[$^{13}CO_2$]-breath tests were performed on day 5 after the start of the experimental diets. Day 5 was chosen for the test since rats need some days to resume their original level of feed intake after being put on an experimental diet. For animals on the 7.5, 10 and 13.8% protein containing diets, all egg white of the main morning meal (at 9:00) was replaced by egg white intrinsically labeled with [1-^{13}C]-leucine. The labeled egg white was produced according to Evenepoel et al. (1997). The amount of the egg white in the morning meals for the 7.5, 10 and 13.8% protein diets was 0.18g, 0.24g and 0.33g, respectively. The absolute amount of egg white in the 5% protein diet (0.12g) was too low to perform an adequate [$^{13}CO_2$]-breath test. Therefore, the 5% protein group was breath tested with [1-^{13}C]-methionine as part of the free amino acid supplement (at 9:00 for the 0 h interval group and at 10:00 for the 1 h interval group). The [1-^{13}C]-methionine breath test was performed by replacing 6.8 mg of the free methionine by [1-^{13}C] methionine (chemical purity > 99%, isotopic enrichment > 99%, Mass Trace, Woburn, USA).

The experimental design with feeding strategy and type of breath test substrate on day 5 is shown in figure 1.

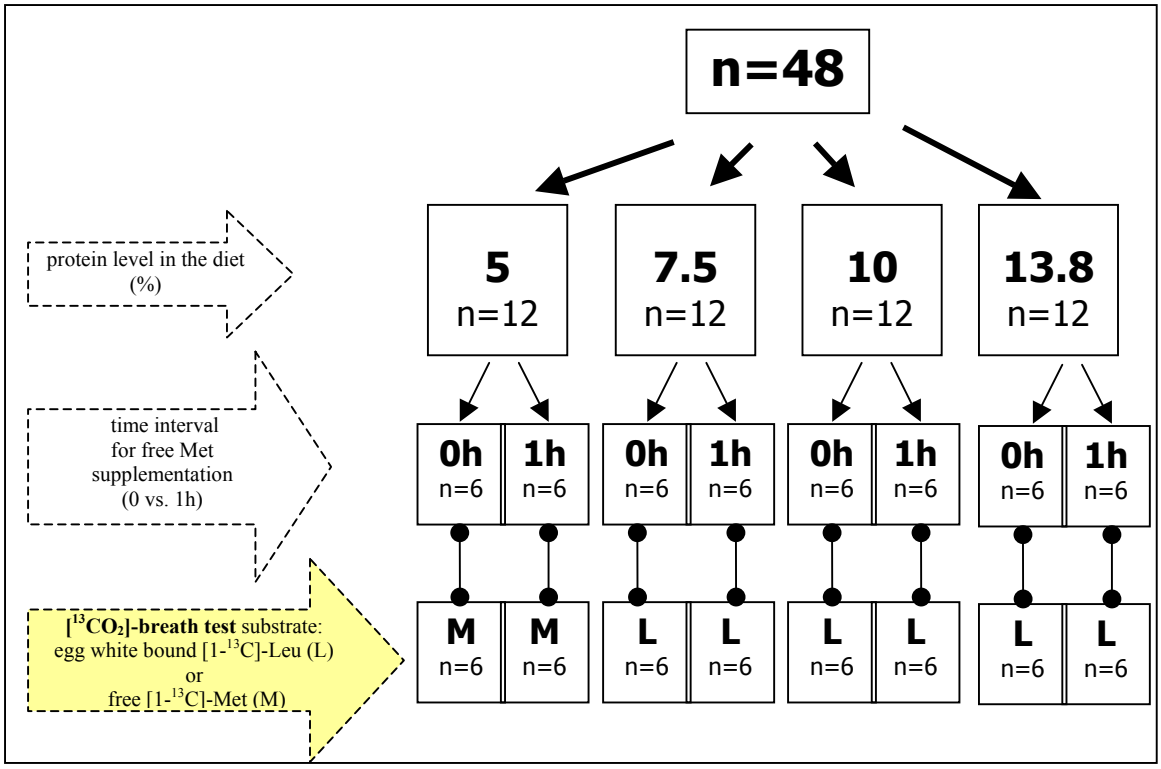

Figure 1. Scheme of experimental design.

To collect expired air during the breath test procedure, rats were placed individually in an airtight macrolon cage with a volume of about 4 liter (20cm*16cm*14cm) bedded with sawdust and *'ad libitum'* access to water. After half an hour a 50 ml air sample was taken from the cage using a syringe fixed on the lid of the case. After each sampling period of 30 minutes rats were placed in a new clean cage with fresh air. The breath collection started half an hour prior to feeding the meal with the label (either labeled protein or in case of the 5% protein diet labeled methionine). The period before the feeding was used to determine the enrichment of expired air before the experimental meal was supplied (null value). Animals received their labeled experimental meal at the normal time (e.g. 09.00 for 30 minutes). In total, 15 breath samples were collected per animal during 7.5 hours (for labeled leucine) or 6 hours (for labeled methionine) of measurements. The collected samples were analyzed by a Finnigan Delta C IRMS (Isotope Ratio Mass Spectrometer, Finnigan, Bremen, Germany) at the WIAS-IRMS facility at the Department of Animal Sciences. The

kinetics of the expiration rate of the label per hour was expressed as percentage of the initial dose of labeled substrate per hour (% dose / h). The total capacity of the rat to expire the label was calculated from the cumulative recovery of label during the entire period of breath collection. The cumulative values were expressed as percentage of the dose expired in 7.5 hours (% dose / 7.5 h) for leucine and in 6 h (% dose / 6 h) for methionine.

Total CO_2-production

In order to calculate the [^{13}C]-expiration rate in breath, values for total CO_2-production were needed. CO_2-production was established as described in chapter 2. In brief, total CO_2-production was measured separately under breath test conditions for some animals in each group. The levels of CO_2-production that were measured, included Diet Induced Thermogenesis (DIT). Since the variation in CO_2 levels is very small compared to change in enrichment, a constant level of CO_2-production per animal was assumed during the period measurements were performed. The mean CO_2 production measured when all animals were combined was 0.14 L CO_2 / 30 min. Values of CO_2-production for individual animals were corrected for differences in Metabolic Body Weight of animals ($W^{0.75}$).

Liver and body composition – day 32

After being fed the experimental diets for 32 days the animals were weighted and sacrificed by exposure to a gas mixture of O_2 and CO_2 (1:2). The carcass of the rats were collected in plastic bags and stored at -20 °C. Carcass were defrosted and the livers were removed. Freezing the rats before removal of the livers prevented excessive blood losses. The livers and the liver free body (including hide, head, feet and tail) were weighted separately. To determine the dry matter (DM) content of the livers, livers were freeze-dried. Dry samples were used for the analysis of fat and nitrogen. Crude fat was determined by extraction with petroleum ether (boiling range 40-60 °C) (Soxhlett-method). After extraction, samples were dried in a vacuum oven at 80°C for at least 2

hours to a constant weight, according to ISO 6492 (ISO, 1985). For crude protein determination nitrogen was estimated using the Kjeldahl method according to ISO 5983 (ISO, 1979). Nitrogen values were recalculated to crude protein (6.25 N).

Liver free bodies (referred to in the text as bodies) obtained from rats fed the highest (13.8%) and the lowest (5%) protein level were examined for DM, fat and nitrogen content. The bodies of the groups on the 10 and 7.5% protein diets were stored again in the freezer at -20°C until further analysis. At the time of analysis these bodies were placed in separate cylinders of known weight and autoclaved for 10h at 130°C and 2 atmosphere with enough water to cover the body. After autoclavation and cooling down, the cylinder with the rat's body and water was weighted just prior to homogenizing and further analysis. For the DM content, samples of the homogenized bodies were dried in a forced air oven at 103°C, according to ISO 6496 (ISO, 1983). For fat analysis body samples were freeze-dried prior to extraction using the same procedure as with the liver samples. For nitrogen analysis wet body samples were used according to ISO 5983 (ISO, 1979). Nitrogen values were recalculated to crude protein (6.25 N).

Statistical analysis

All data for fat and crude protein (CP) were expressed as percentage of dry matter (DM). The MANOVA and post hock LSD test (Statgraphics Plus, version 4.1) was used to determine statistically significant differences. Values were considered to be significantly different when $p < 0.05$. Values are expressed as mean $\pm$ SD.

RESULTS

$^{13}CO_2$ breath test – day 5

The rate of [^{13}C]-expiration from orally ingested labeled substrates with the morning meal was monitored as [$^{13}CO_2$] exhaled for 7 hours until half an hour before the afternoon meal at 16.30 (6 hours for the group that received labeled methionine in the supplementary meal with 1 hour interval). Values are

expressed as percentage of the original dose (provided at 0 or 1 h interval) per hour.

Figure 2 shows the expiration rate of label from meals containing egg white bound [1-^{13}C]-leucine in methionine deficient protein meals containing 7.5, 10 or 13.8% protein.

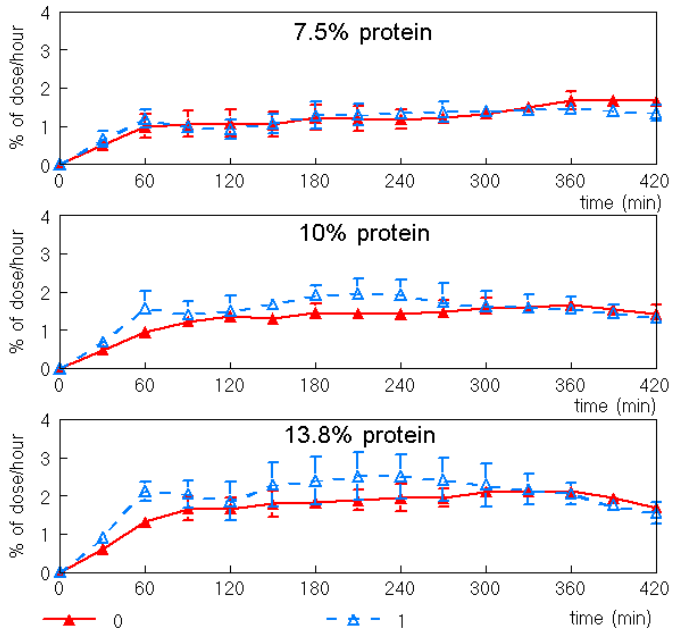

Figure 2. Rate of ^{13}C expiration (% of dose/hour ±SD) derived from egg white bound [1-^{13}C]-leucine (n=6 for each interval group) on day 5 after the start of the experimental diet. 0 (filled triangles) and 1h (open triangles) groups received meal followed by the free methionine supplement. Free methionine was either given together with the main meal or after 1 hour.

Figure 2 shows that the label expiration rate from egg white bound [1-^{13}C]-leucine was detectable after 30 minutes. The pattern of the expiration rate was similar for all 0 hour interval groups on the three diets containing 7.5, 10 or 13.8% protein. The rate of label expiration was highest in animals fed the 13.8% protein diet. The expiration rate increased to a maximal value after about 60 - 90 minutes. Thereafter, a more or less constant plateau value was maintained. Until the end of the measurements after 7 hours (half an hour before the evening meal was given), the [^{13}C] expiration rate was still not back to zero.

The expiration rate patterns for the animals that were fed the free methionine 1 hour after the morning meal followed more or less the same pattern as for the 0 hour interval studies. Animals given the 7.5 % protein diet appeared to have no difference in recovery between 0 and 1 h.

The expiration rate pattern was similar for all 1 hour interval groups independent of the protein levels in their diet. The rate of label expiration was the highest in animals fed the 13.8% protein diet. The expiration rate increased to a maximal value after about 1 hour. Thereafter, a more or less constant plateau value was maintained, with some fluctuations. From 3.5 hour onwards, the label expiration rate gradually declined. This decline is slightly more pronounced in animals fed the 13.8% protein diet. Until the end of the measurement period, like in the 0 h groups, the [^{13}C] expiration rate was still not back to zero.

The plateau values in the groups that were fed a combination of the main meal (10 or 13.8 % protein) and free methionine tended to be slightly lower compared to the groups that received free methionine 1 hour after the main meal These differences were more pronounced in the animals fed the diets with a higher protein content.

Values for the cumulative recovery of label from egg white bound [1-^{13}C]-leucine are presented in table 2. The cumulative values over 7 hours differ for the three protein levels in the diets, with higher values for the diets with a higher protein content. The cumulative recovery for the 7.5% protein diet was similar when main meal and free methionine were given together or when methionine was given at a 1 hour were similar. In addition, differences in cumulative values between the 0 and 1h interval groups in the 10% protein diet groups were not significantly different. Cumulative label recovery in rats fed the 10% protein diet in combination with free methionine did not significantly differ from both animals fed the 7.5% protein diet to which free methionine was either given immediately or after 1 hour time interval. Moreover, the cumulative recovery in the rats fed the 10% protein diet to which free methionine was

given with a 1 h time interval was not different from 13.8% with a 0 hour interval. A significantly different cumulative label recovery was only noticed in rats fed the 13.8% protein diet with a higher recovery rate when the methionine supplement was provided with a 1 hour time interval.

Figure 3 shows the rate of [^{13}C]-expiration from free [1-^{13}C]-methionine supplemented to the 5% protein diet.

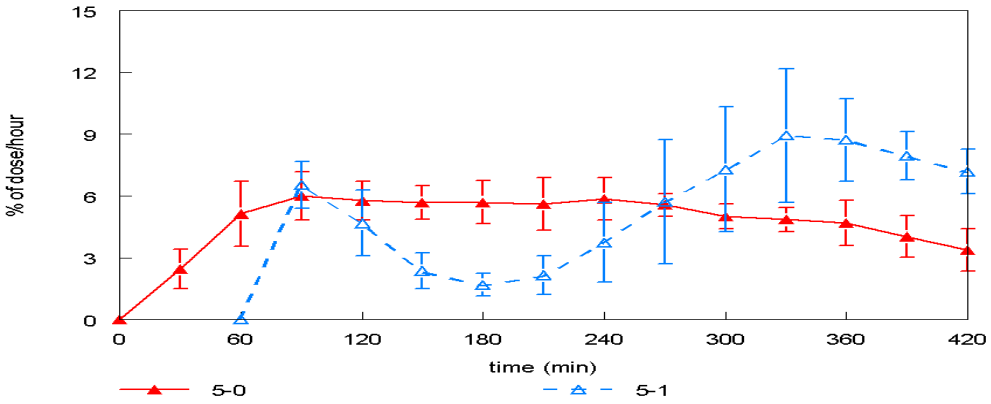

Figure 3. Rate of ^{13}C expiration (% of dose/hour ±SD) derived from free [1-^{13}C]-methionine (n=6 for each interval group) at day 5 after the start of the experimental diet. 0 (filled triangles) and 1h (open triangles) groups received the main deficient protein meal followed by the free mehionine supplement either together with main meal or after 1 h.

The label expiration rates of free [1-^{13}C]-methionine in the supplements showed completely different patterns for the 0 and 1 hour interval groups. In case the methionine was mixed with the 5% protein meal, the expiration rate curve showed a pattern comparable to the label expiration rate from egg white bound [1-^{13}C]-leucine (figure 2). It increased to the level of approximately 6% after 90 minutes and this plateau value was kept for the next 3 hours, after which the rate of label expiration slightly decreases to approximately 3.4% at the end of measurement period (7 hours).

The expiration rate of label administered with the supplement 1 hour after the main meal reached a level of 6.5% per hour 30 minutes after ingestion of the supplement. The expiration rate pattern was biphasic over the entire period air samples were collected. Between 1 hour and 2 hours after the supplement was given the expiration rate decreased to a minimum value of 1.7% ± 0.5. From this point in time onward the expiration rate increased again to reach a second peak value of 8.9% ± 3.3 after 4.5 hours. By the end of the air sample collection period after 6 hours the expiration rate had declined to a value of 7.2% ± 1.1. The cumulative recovery of label from free [1-^{13}C]-methionine after 6 hours of measurements is shown in table 2. The differences between both time intervals (31% ± 3.3 for 0 hour and 33% ± 6.5 for 1 hour interval) were not significantly different.

Table 2: The cumulative recovery of label from egg white bound [1-^{13}C]-leucine after 7 hours of measurements and from free [1-^{13}C]-methionine after 6 hours of measurements on day 5 after the initiation of the diets. Groups of animals were fed diets with different protein levels. Free methionine was either mixed with the main experimental meal or supplemented 1 hour after the main meal. Significant differences between groups ($p<0.05$) are indicated by different letters.

Dietary protein level [%]	[1-^{13}C]-leucine		[1-^{13}C]-methionine	
	0 h interval	1 h interval	0 h interval	1 h interval
5			31.2±3.3^{a}	33.5±6.5^{a}
7.5	8.7±1.4 ^{A}	8.6±1.0 ^{A}		
10	9.5±0.7 ^{AB}	11.0±1.6 ^{BC}		
13.8	12.3±1.0 ^{C}	14.1±2.2 ^{D}		

Body weight gain – day 32

All the animals were weighed daily, prior to the main morning meal at 09.00 hour.

At day 0 when the animals were put on the experimental diets the mean weight of the animals was 141g (sd=7). The weight gain over 32 days (figure 3) was influenced by both the protein level of the diet ($p<0.0001$) as well as the time interval of free methionine supplementation ($p=0.0242$). There was also an interaction observed for weight gain between protein level and the time interval ($p=0.0002$). The lowest weight gain over 32 days was observed in the group fed the 5% protein diet. Within this group a significantly higher weight gain was observed in the 1h interval group (35 g, sd=4) compared to the animals that were fed the mixture of the protein deficient diet together with the free amino acid supplementation (29 g, sd=5). All animals fed the diets that contained the higher protein levels (7.5, 10 or 13.8%) showed a significantly higher weight gain compared to the animals fed the 5% protein diet. After 32 days on the diet there was no difference in weight gain between the group fed the 7.5% diet mixed with free methionine or the animals that received the free methionine supplementation 1 hour later. In the groups fed the 10 and 13.8% protein level diets, the weight gain was lower in the groups that received methionine supplementation 1 hour after the main meal compared to 0h interval groups. The adverse effect on weight gain caused by the 1h supplementation interval was significantly higher in the group fed the 13.8% protein diet. The weight gain in both the 0 hour and 1 hour interval groups fed the 10% protein diet was higher than the weight gain in the 1h interval group fed the 13.8% protein diet.

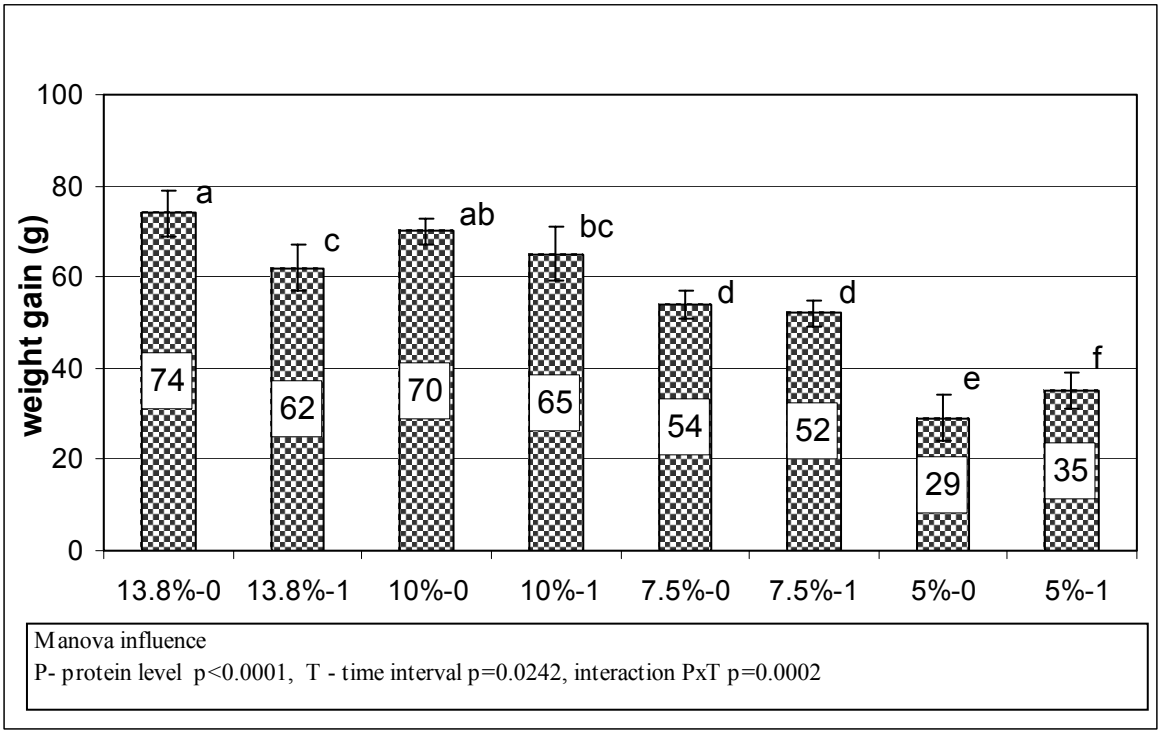

Figure 4. Body weight gain (g) in rats 32 days fed the experimental diets of 13.8%, 10%, 7.5% or 5% protein and 50% deficient in methionine. Free methionine was supplemented after 0 or 1h after the main meal was served. Significant differences between groups ($p<0.05$) are indicated by different letters.

Liver and carcass composition

◇ *body without the liver (hereinafter referred to as "body")*

The DM content was slightly higher in animals fed the 5% protein diet and supplemented free methionine with an 1h interval (34.4 ± 1.0% of body weight) compared to the other 3 treatments. Mean values of DM for these 3 treatments were 32.5 ± 1.7%, 31.6 ± 1.3%, 32.7 ±0.7% for 5%-0, 13.8%-0 and 13.8%-1 groups, respectively.

DM composition of the bodies of animals on the 5 and 13.8 % protein diets are shown in figure 5.

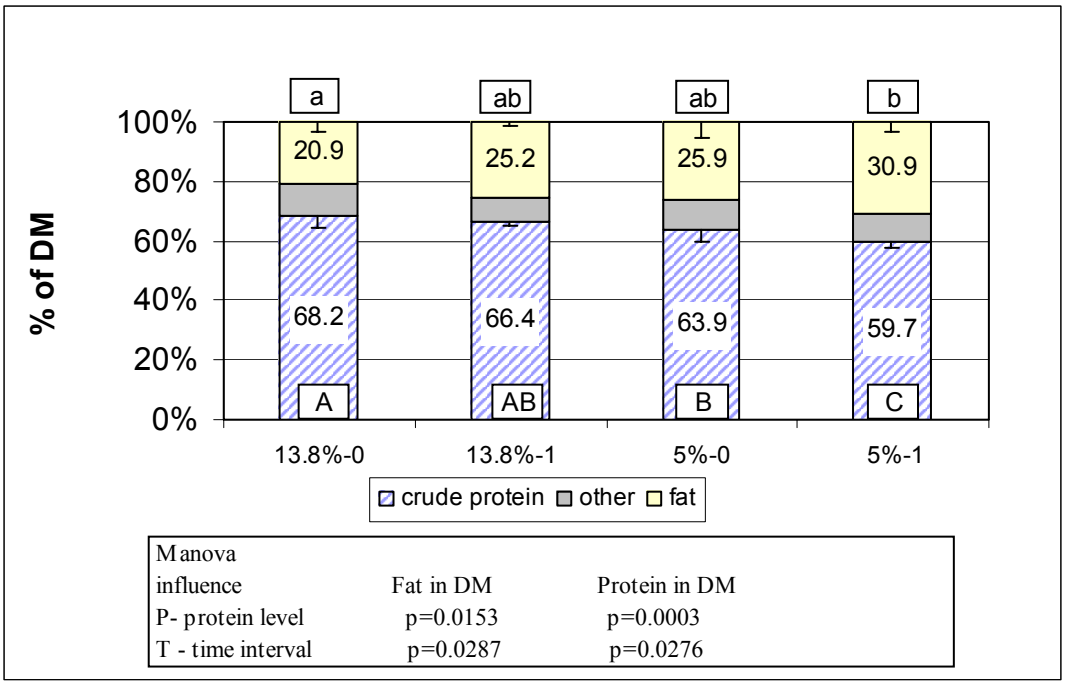

Figure 5. Dry matter composition of bodies without the liver of rats after being 32 days on either a 13.8% or 5% protein diets deficient in methionine and supplemented with free methionine at 0 or 1h after the main meal was served. Significant differences between groups ($p<0.05$) are indicated by different letters (small letters for fat and capital letters for protein).

Both, the protein level of the diet and the time of free methionine supplementation influenced fat and protein content in the body DM. The highest percentage of fat (30.9 ± 3.2% of DM) was found in the animals fed the 5% protein diet in which free methionine was given at a 1h time interval. The difference was significant compared to the fat content in the 13.8% protein diet group receiving the mixed diet (0 hour time interval (20.9 ± 3.4% of DM)). The share of the protein content in the body was opposite to that of fat with the highest protein content in the 13.8% protein diet group with 0h interval (68.2 ± 3.6% of DM). The value was significantly higher compared to the 0 and 1 hour interval groups fed the 5% protein diet. The lowest body protein content was observed in rats fed the 5% protein diet to which methionine was given with a 1 hour time interval (59.7 ± 1.9% of DM) and it was significantly lower compared to all analyzed groups.

◇ *livers*

DM composition of the livers for all 8 experimental groups is shown in figure 6. The highest DM content was observed in animals fed a 5% protein diet with 26.7 ± 1.4% as the mean of both (0 and 1 hour) interval groups. The lowest DM content in liver was found in rats fed a 7.5% protein diet (mean for both the 0 and 1 hour interval group was 24.9 ± 0.5%). The DM content in the 10 and 13.8% protein diet groups was 25.2 ± 0.5% and 25.6 ± 0.6%, respectively.

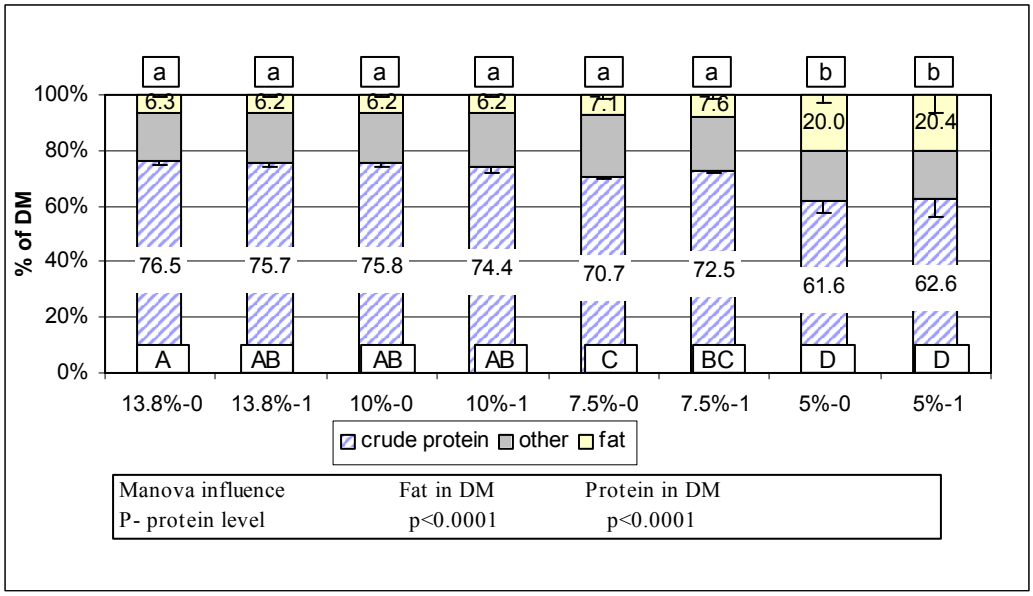

Figure 6. Dry matter composition in rats' livers 32 days after being fed a diet of 13.8%, 10%, 7.5% or 5% protein supplemented with free methionine after 0 or 1h time interval following the main meal. Significant differences between groups ($p<0.05$) are indicated by different letters (small letters for fat and capital letters for protein).

The protein level of the diets had a significant influence on the fat and protein content of the DM in the livers. The highest percentage of fat was found in livers of the animals fed the 5% protein diet (20.2 ± 4.7% of DM). This was significantly higher compared to all other groups fed the diets with different protein levels (with 7.4 ± 1.3%, 6.2 ± 0.5%, 6.2 ± 0.5% of fat content respectively for the 7.5, 10 and 13.8% protein diets).

The protein content in the livers was higher in the animals fed the 13.8% protein diet ($p<0.001$). The content of protein in the liver of animals fed the 5% protein diet (62.1 ± 5.0%) was lower than in all other groups. In the group fed the 7.5% protein diet the protein content in the livers was also different from the levels observed in the other dietary groups. The mean protein content in the group fed the 7.5% protein diet was 71.6 ± 1.4% for both the 0 and 1 hour time interval group. The highest protein content (76.1 ± 1.4%) was observed in the livers of the group fed the 13.8% protein diet as well as in the group fed the 10% protein diet (75.1 ± 2.1%).

DISCUSSION

The present study examined the influence of the time interval (0 or 1 hour) of single free methionine supplementation to animals fed a diet of different protein content that was 50% deficient in methionine, on post prandial dietary amino acids oxidation, weight gain and carcass composition.

$^{13}CO_2$ breath test

Leucine was chosen as a tracer in the $^{13}CO_2$ breath test analysis, because this indispensable amino acid is used in the body only for protein synthesis. The leucine that is not used for protein synthesis will be decarboxylated. The decarboxylated part allows assessing the part of dietary amino acids that is lost for protein metabolism (Reeds and Davis, 1999).

In the present study the time interval between supplying the methionine deficient meal and its free methionine supplement did influence the post prandial oxidative losses of dietary amino acids at diets containing protein levels above 7.5 %. This finding is similar to that was observed in other studies (Gas et al., chapter 2).

The label expiration rate from egg white bound [1-^{13}C]-leucine was already detectable 30 minutes after the meal was given. The expiration rate increased to a maximal value between 60-120 minutes after the meal was supplied. Thereafter, a more or less constant plateau value was maintained. The pattern

of label expiration rate was similar for the three diets with protein levels of 7.5, 10 or 13.8 % independent of the time interval at which free methionine was supplemented (0 or 1h after the main meal). However, when the diet contained a higher protein level, the oxidative losses of the label were higher. In animals fed the 13.8% protein diet also differences between the 0 and 1h time interval of methionine supplementation became visible.

The collecting of the air samples was stopped after six or seven hours, half an hour before the evening meal. At that time the [^{13}C]-expiration rate had still not returned to zero. In case of the animals fed the 7.5% protein diet, the label expiration rate from [1-^{13}C] egg white bound leucine for both the 0h and 1h time interval groups exhibited more or less a similar pattern. The expiration rate level was higher in the groups fed the 10 and 13.8 % diets in which free methionine was supplemented with a 1h time interval. This was also illustrated by the values of cumulative label recovery. No significant difference between the 0 and 1 hour time interval of methionine supplementation was observed in case of the 10% protein diet.

The label expiration rates of free [1-^{13}C]-methionine measured in animals fed the 5% protein diet showed a totally different pattern 0 hours compared to the group that was supplemented with methionine at the 1 hour time interval (figure 3). The 0 hour interval group showed a similar pattern of [1-^{13}C]-methionine expiration compared to the pattern observed for [1-^{13}C]-leucine in the groups fed the 7.5, 10 or 13.8% dietary protein levels. The 1 hour interval group showed a bi-phasic expiration rate of the labeled methionine. The cumulative recovery of [1-^{13}C]-methionine was about 3-times higher than the cumulative recovery of labeled leucine. Assuming that leucine is a good indicator for protein synthesis, one has to keep in mind that a substantial part of dietary methionine is not used for protein synthesis but is used for other functions. Nevertheless, the amount of methionine supplemented to the diets was based on daily requirement data (NRC, 1995). The high oxidative losses of methionine may be explained by non-protein functions of methionine.

Methionine beside its direct role in protein synthesis serves as a sulfur donor in case of cysteine synthesis and as methyl donor in various other reactions (Finkelstain 1998). The transsulfuration pathway leads to the release of $^{13}CO_2$ from [1-^{13}C]-methionine (measured with [1-^{13}C]-breath test) and may thus explain the high oxidation rate of methionine (Raguso et al. 1999). In this study, the cumulative recovery of [1-^{13}C]-methionine did not significantly differ between intervals but the difference in the pattern was remarkable. Body weight gain was significantly higher when methionine was supplemented 1 hour after the main meal. Apparently, the high level of methionine oxidation does not interfere with body weight gain.

Body weight gain

Animals gained weight proportionally to protein level in the diet. Only in the group fed the 13.8 % protein diet the weight gain was not significantly different from the animals fed the 10 % protein diet. Overall, the interval of free methionine supplementation also affected weight gain. In the animals fed the 13.8 % protein diet mixed with free methionine weight gain was higher compared to the group that received methionine 1 hour after the main meal. These observations are in agreement with post prandial catabolism which at these protein levels was higher when free methionine was supplemented at an 1 hour time interval. This suggests that there may be an advantage in feeding free amino acid at the same time as the main meal. When animals were fed the 5 % protein diet mixed with free methionine, however, weight gain was lowest of all experimental groups. Although it was hypothesized that the impact of the supplementation strategy on body weight gain would be more clear in animals fed the lower protein diets, these differences were noticed in both the low and high protein level diets (13.8% and 5%).

Four different protein levels (5, 7.5, 10 and 13.8%) in the diets were chosen and two time intervals for single free methionine supplementation: 0h as standard approach in practice (mix with deficient meal) and 1h as experimental approach (chosen due to the results of the study described in

chapter 2). All diets were isocaloric (ME = 13.4 kJ/g). The energy difference due to the variation in dietary protein content of the diets was compensated for by an equivalent change in dietary carbohydrate content. The food intake level was set as high as that of the 5% protein group, which had the lowest intake throughout the experiment. Therefore, all experimental groups had their feed intake restricted to an average of 11g a day during the 32 days of the experiment. It has been shown that rats increased their food intake with moderate protein restriction (8-10% of dietary protein), and decreased their food intake under more severe (5%) dietary protein restrictions (Du et al. 2000). Some other studies also showed that diets with a low protein content depressed food intake (Beck et al. 1989, Mercer et al. 1994). In contrast several other groups found increased food intake in case of low protein diets (Colombo et al. 1992, Deschepper and de Groote 1995, Swick and Gribskov 1983, White et al. 1994 and 1998)

When protein utilization was measured using the protein efficiency ratio (PER), which is the ratio of body weight gain to protein consumption, it became clear that in case of the diet with the highest protein level (13.8%) there was a relatively lower protein efficiency (PER=1.5 in case free methionine was supplemented 1 hour after the main meal, and a PER=1.3 when main meal and free methionine were mixed). The low efficiency in dietary protein utilization can be explained by the relative low level of feeding. Because energy intake was similar in all treatment groups, energy may have become limiting for weight gain at a high dietary protein level. Interestingly, in case of the 5% protein diet with 0 hour time interval, protein efficiency was lower (PER=1.6) than in case of the 7.5 and 10% protein diets where the PER was intermediate (PER=2.0).

The body weight gain results were in line with the breath test results within each dietary protein group. Due to the higher cumulative oxidative losses of label in the 1 hour interval groups fed the 13.8% and 10% protein diets respectively, growth was reduced.

Body composition

Four groups of animals (fed either 13.8 or 5% protein diet, either mixed with free methionine or to which methionine was supplemented after 1 hour) were chosen for the analyses of body composition without the liver (the livers were analyzed separately). In case of the high dietary protein level (13.8%), there was more protein and less fat content present in the DM compared to animals on the 5% protein diet. These observations confirmed earlier findings of an increase in body fat content in the rats fed low protein diets (Meyer, 1958; Noblet et al., 1987; Rothwell et al., 1983; Swick and Gribskov, 1983; White et al., 1994 and 1998; Du et al., 2000). Obviously, this is caused by the fact that diets with lowered protein content were compensated for by extra carbohydrates in order to keep the diets iso-energetic. This caused high level of lipogenesis on low (5%) dietary protein level. Therefore, it was understandable that the body composition showed a lower protein content and more lipid deposition in dry matter in rats fed the 5% protein diet compared to the 13.8% protein diet.

The livers were analyzed for all animals. Again lower levels of protein in the diet resulted in a higher fat content though this only reached the level of significance in animals fed the diet with the lowest (5%) protein content. The high fat content in the livers of the animals fed the 5% protein diet suggested impairment in fat metabolism in the livers. In case of such a protein deficient diet, severe methionine deficiency as methyl donor is expected. Methionine is an essential amino acid (AA) that besides its role as a substrate for body protein synthesis is a major source of methyl groups for a number of important reactions (Cantoni G.L. et al. 1980 and 1982). It has for instance been reported that dietary methionine is a precursor in the biosynthesis of choline (Eckstein, 1952). Choline deficiency induces severe fatty infiltrations in the liver (Aoyama.et al. 1971). This can be cured by restoring methionine levels in the diet, or alternatively by administering choline, which the body would normally form from methionine (Mauro Di Pasquale M.D. 1997). It is possible that due to the reduced choline production as a consequence of methionine deficiency

caused accumulation of triacyloglyceroles in the liver (Aoyama.et al. 1998). In addition, methionine together with lysine are substrates for carnitine synthesis (Bremer J. 1983). Metabolically carnitine plays a very important role, as a carrier of long-chain fatty acids into the mitochondrion where it can be used for β-oxidation. In practice, carnitine enables fatty acids to be used as a source of energy (Hoppel C.L. 1982). Consequently, impairment of all the methionine related functions leads to accumulation of fat in the livers of rats fed very low protein diets and as a consequence low methionine diets.

The time interval of 1 hour for free methionine supplementation in the present study did not influence neither fat nor protein content in the livers.

CONCLUSIONS

From the results presented in this study, it can be concluded that:

1. Increased [1-^{13}C]-leucine oxidation is in line with an increase in the dietary protein level (7.5, 10 or 13.8%)
2. Methionine oxidation was considerably higher than leucine oxidation, indicating that less methionine compared to leucine is deposited in body protein.
3. The kinetics of methionine oxidation were influenced by the time interval of its supplementation, though the cumulative oxidation stayed the same for the 0 and 1 hour time intervals.
4. The 1 hour time interval for free methionine supplementation influenced growth differently depending on the dietary protein level.
5. Animals fed a severely protein deficient diet (5%) benefited from introduction of an 1 hour time interval for supplementation of free methionine in terms of weight gain.
6. Dietary protein deficiency caused fatty livers but only when the dietary protein content was lower than 7.5%.
7. The increase in protein content in the diet resulted in better growth of the animal despite the lower post prandial amino acid utilization, as was measured by leucine oxidation.

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Chapter 4

Impact of lysine and/or methionine deficiency on body weight gain and on body and liver composition in growing rats.

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ABSTRACT

The present study examined body weight gain as well as body and liver composition in rats fed a lysine and methionine deficient protein diet (15 and 10%) without any supplementation or supplemented with free lysine and/or methionine. The body composition analysis included dry matter (DM), fat and nitrogen content in the livers and remaining carcasses.

Lysine deficiency reduced body weight gain in rats fed either the 15 or 10% protein diets. Methionine deficiency did not have a significant impact on weight gain but tended to increase the fat content of the livers (both in case of the 15% and 10% protein diets) probably via affecting the choline status in the body.

An interaction between the protein level of the diet and free amino acid supplementation strategy was observed. Methionine supplementation, in the presence of the lysine supplement, tended to decrease body weight gain in case of the 15% protein diets but caused an increase in weight gain in rats fed the 10% protein diet.

The results showed also a significant influence of free amino acid supplementation on the fat content of the livers. Lysine and methionine deficiency increased the fat deposition in the livers compared to situation when both amino acids were supplemented both in case of the 15% and 10% protein diets. This could be explained by methylation of lysine to carnitine by methionine. The availability of carnitine makes the use of fat for energy expenditure possible. In case of a shortage of amino acids required for carnitine synthesis, the use of fatty acids for energy expenditure was affected. The protein level in the diet influenced fat content in the bodies as well. Animals fed the 15% protein diet had a higher body fat content than animals fed the 10% protein diet.

Keywords: lysine, methionine, free amino acid supplementation

INTRODUCTION

Poor quality proteins in animal nutrition, mainly from vegetable origin, are commonly supplemented with free amino acids to meet the recommended amino acid pattern. The ideal amino acid pattern is often based on body composition. It has been suggested by Baker (1991) that lean body mass is a major predictor of nutrient requirement in beef heifers. Other groups have suggested that microbial indispensable amino acid *de novo* synthesis contributes considerably to meet the amino acid requirements of the body (Metges 1999 and 2000, Backes 2002). The amino acids lysine and methionine are often the limiting amino acids when considering the requirements of the body (Mauro Di Pasquale M.D. 1997). It should be kept in mind, however, that these amino acids have also other specific functions in addition to contributing to protein synthesis. Lysine is for instance one of the precursors of carnitine and has been shown to have a positive effect on Calcium balance (Civitelli R. et al. 1992). Methionine is an essential amino acid that is required for protein synthesis and also acts as a source of sulfur for cysteine synthesis and methyl groups for a number of important reactions such as synthesis of choline, creatine, carnitine and both DNA and RNA intermediates (Cantoni G.L. 1980 and 1982). Dietary deficiency of methionine in rats results in a fatty liver, a reversible process which can be cured by restoring the level of this amino acid in the diet, or alternatively by giving choline, which the body would normally form from methionine (Mauro Di Pasquale M.D. 1997). It has been suggested that due to a reduced choline production as a consequence of methionine deficiency, triacyloglyceroles accumulate in the liver, resulting in a fatty liver (Aoyama et al. 1998). Both lysine and methionine are precursors for carnitine synthesis (Bremer J. 1983). Metabolically, carnitine plays a very important role, as it serves as a carrier of long-chain fatty acids into the mitochondrion where it can be used for β-oxidation. In practice, carnitine enables fatty acids to be used as a source of energy (Hoppel C.L. 1998).

In the study described in chapter 2, we have found that methionine supplementation of protein diets 50% deficient in methionine, influenced post

prandial leucine oxidative losses, however, without having any impact on body weight gain. Therefore, it was assumed that the amino acid pattern in case of a 50% methionine deficient diet was still adequate for rats to allow normal growth to occur. The aim of the present study was to investigate to which extent dietary deficiency in lysine and methionine affected weight gain in rats. It was also studied whether deficiency in these two amino acids affected body composition, in particular the livers with respect to fat and nitrogen content.

MATERIALS AND METHODS

Animals

This study was performed with 48 male, 5-6 week old Wistar (WU) rats (Harlan, Horst, The Netherlands) with an initial weight of 100-125g. The Committee for Animal Welfare of Wageningen University approved the experiments in the present study.

Housing

The rats were housed individually in macrolon cages (38 x 26 x 14 cm) in the animal facilities of Wageningen University at controlled temperature (21^{o}C) and relative humidity (70%). The daily light schedule included 8 hours of darkness and 16 hours of light. For convenience of feeding, the dark period was set during day time (09.00 - 17.00). Water was available 'ad libitum'. The animals were after arrival first familiarized with their new environment for 2 weeks before the actual treatments started.

Feeding and diets

During the period of conditioning to new environment period, commercial rat chow (Teklad Global Rodent Diet; Harlan, The Netherlands) was provided to the animals. To keep the energy intake at the same level for all animals the feed supply was gradually increased from 5 g to 11 g a day depending on the intake of the previous day. After the pre-conditioning period, the animals were

assigned to one of the eight experimental diets. There were 2 major groups fed semi-synthetic and isoenergetic diets containing either 15% or 10% of protein. The 15% protein diet was chosen because the National Research Council (NRC) (1995) recommends that level of protein for growing rats. The 10% protein was expected to show some deficiencies in the amino acid pattern. The two diets were 45% deficient in the amino acid lysine and 34% in methionine when taken into account the tabulated pattern of amino acid requirement for growing rats (NRC, 1995). The content of the other nutrients was in line with the requirements of the NRC. Both protein level groups were further divided into 4 subgroups where the animals were fed the corresponding experimental diet without or with lysine and/or methionine supplemented as free form amino acids mixed with the main meal. This resulted in the feeding schedule presented in figure 1.

- Complete amino acid pattern in the diet, the protein content of the diet being 10 or 15%, supplemented with both deficient amino acids, lysine and methionine ("L/M" groups) (n=6)`
- Methionine deficient diets with a protein level of 10 or 15 %, supplemented with lysine only ("L/-" groups) (n=6)
- Lysine deficient diets with a protein levels of 10 or 15 %, supplemented with methionine only ("-/M" groups) (n=6)
- Diets with a protein level of 10 or 15 % deficient in both lysine and methionine (no amino acid supplements added, "-/-" groups) (n=6)

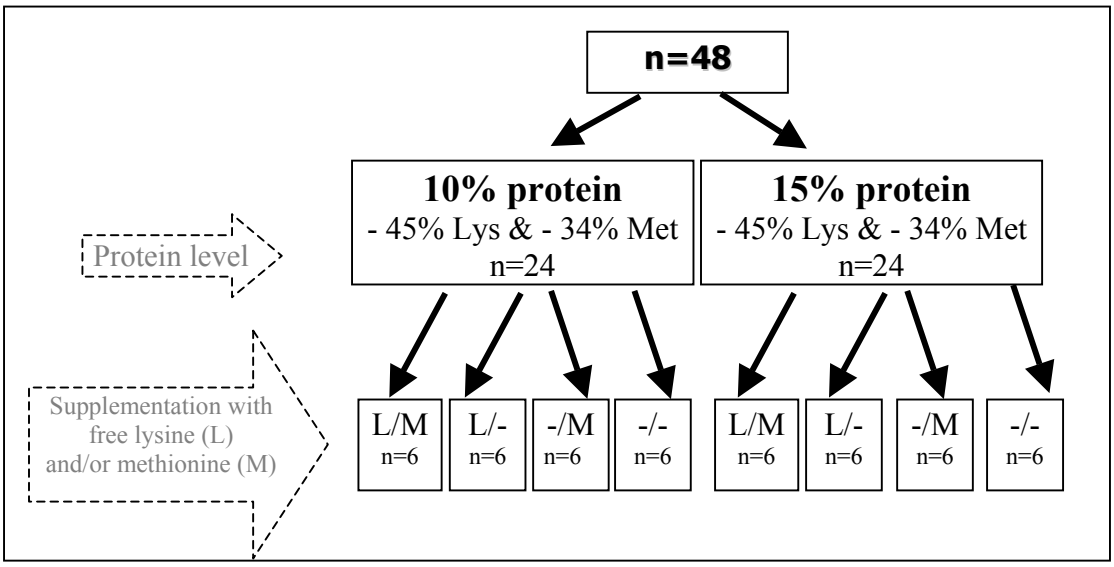

Figure 1. Experimental diet design. Abbreviations: L=lysine, M=methionine

The animals were fed twice a day during 30min at the start and the end of the dark period (09.00 - 09.30 and 16.30 - 17.00). The feed intake was gradually increased during the experimental period of 19 days from 10 g to 17.5 g per day.

The composition of the diets containing 15 or 10% protein is shown in table 1. Protein levels of the diets were modified as a result of the addition of free lysine and/or methionine.

Table 1. Composition of the diets. Values in parenthesis apply only to the diets to which free lysine and/or methionine were added.

Feed ingredients (g/kg):	**15% protein diet**	**10% protein diet**
Wheat gluten	73.0	48.7
Chicken egg white powder	50.0	33.3
Wheat	400.0	266.7
Wheat starch	279.6	437.3
L-lysine HCl	0.0 **(5.3)**	0.0 **(3.5)**
DL-methionine	0.0 **(3.4)**	0.0 **(2.3)**
Calculated values (g/kg)		
Net Energy (kcal/kg)	3 282	3 281
Crude protein	157	106
Crude fat	40	37
Lysine	5.1 **(9.2)**	3.4 **(6.2)**
Methionine	3.3 **(6.7)**	2.2 **(4.5)**
Methionine + Cystine	6.4 **(9.8)**	4.3 **(6.5)**

Measurements

Rats were weighted daily before the first meal was given at 9:00. After being fed the experimental diet for 19 days, the animals were sacrificed using an O_2/CO_2 gas mixture (ratio 1:2). The carcass of each rat was collected in a separate plastic bag and stored at -20 °C. Subsequently, the carcasses were defrosted and the livers were removed from the bodies. Freezing the bodies before removing the livers prevented excessive blood losses. The livers and the liver free bodies (including hide, head, feet and tail) were weighted separately for analysis of dry matter (DM), fat and protein content. These analyses were carried out as described in chapter 3. All data for fat and crude protein (CP) in the livers and the liver free bodies were expressed as percentage of DM.

Statistical analysis

The MANOVA variance analysis and post hock LSD test (Statgraphics Plus, version 4.1) were used to determine statistically significant differences ($p < 0.05$). Values are expressed as mean $\pm$ SD.

RESULTS

Weight gain on day 19

At the stat of the experiment the body weight of the animals was 149 g $\pm$ 6. After 19 days of experimental feeding (figure 2) the body weight gain was highest in the 15% protein methionine deficient diet group (15% L/-) with a mean weight gain of 92 g. A slightly lower weight gain of 90 g was observed in the group fed the 15% protein diet supplemented with both amino acids (15% L/M). The weight gain in this group was similar to the weight gain in the animals fed the 10% complete protein diet. The lowest weight gain, 64 g, was observed in the rats fed the 10% protein diet deficient in lysine and methionine (10% -/-). The weight gain of this group was slightly improved when methionine was supplemented to this diet (10% -/M).

An interaction between protein level and supplementation strategy was observed. Methionine supplementation tended to decrease the weight gain in

rats fed the 15% protein diets and to increase the weight gain in the animals fed the 10% protein diet (figure 2).

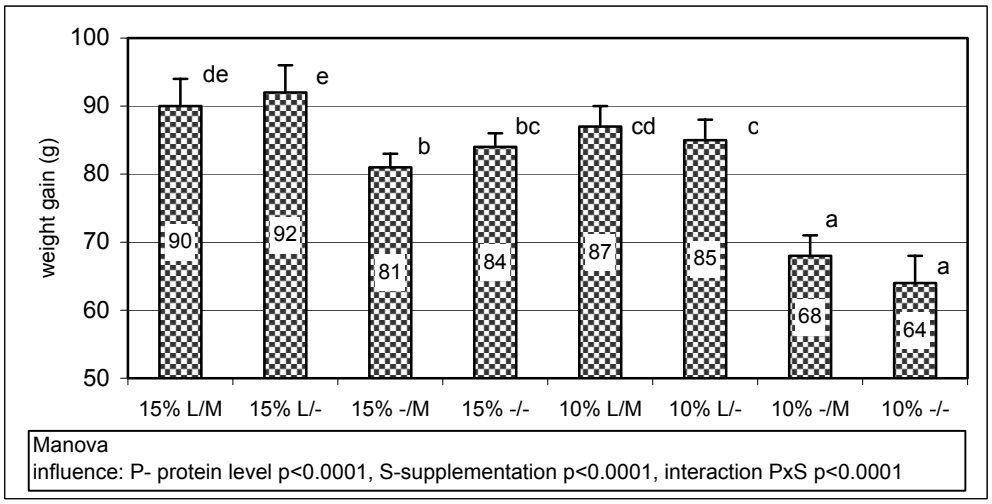

Figure 2. Body weight gain (in g) of rats after being 19 days fed a 15% or a 10% protein diets lysine and methionine deficient, supplemented with free lysine (L) and/or methionine (M). Significant differences between groups (p<0.05) are indicated by different letters.

Table 2 Relation between lysine content in the diet and body weight gain. Body weight gain (given from the highest to the lowest) after being fed for 19 days a 15% or 10% protein diet deficient in lysine and methionine without any amino acid supplement or supplemented with free lysine (L) and/or methionine (M). Significant differences between groups (p<0.05) are indicated by different letters.

#	weight gain after 19 days (g)		calculated amounts g/kg in the diet		experimental group
	mean	±sd	Lys	Met	
1	92^{e}	4	9.2	6.4	15% L/-
2	90de	4	9.2	9.8	15% L/M
3	87^{cd}	3	6.2	6.5	10% L/M
4	85^{c}	3	6.2	4.3	10% L/-
5	84^{bc}	2	5.1	6.4	15% -/-
6	81^{b}	2	5.1	9.8	15% -/M
7	68^{a}	3	3.4	6.5	10% -/M
8	64^{a}	4	3.4	4.3	10% -/-

Body composition

Liver free

DM composition of the liver free bodies is shown in figure 3.

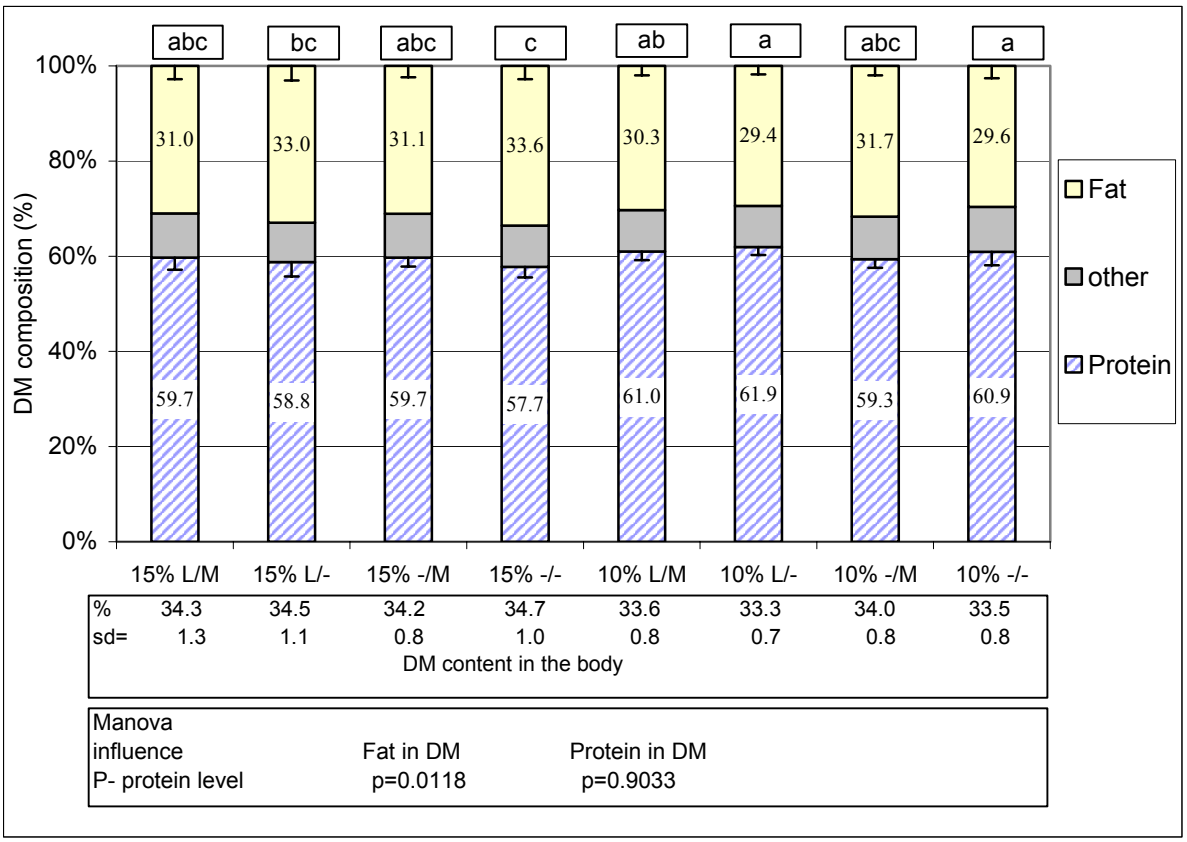

Figure 3. Dry matter (DM) composition and content in the body of rats (without the liver) fed for 19 days a 15% or 10% protein diet deficient in lysine and methionine, without amino acid supplement or supplemented with free lysine (L) and/or methionine. Significant differences between groups ($p<0.05$) are indicated by different letters.

The mean DM content in the body of rats fed the 15% protein diet was 34.4% ± 1.0 whereas in animals fed the 10% protein diet this was 33.6% ±0.8. The percentage of fat content in the body DM was influenced by the dietary protein level but not by supplementation with lysine or methionine. In the rats fed the 15 % protein diet a higher body fat deposition was with a mean value of 32.2% ± 2.8 while in the animals fed the 10% protein diet the mean body fat content was 30.3% ± 2.2.

Methionine deficiency of the 15% protein diet slightly increased the body fat content in DM. In contrast, in animals fed the 10% protein diet, methionine deficiency slightly decreased the body fat content in DM.

The protein content in the body ranged from 57.7 to 61.9% of DM and was not significantly influenced by the protein level in the diet. Supplementation with free amino acids did neither influence protein content nor fat content in DM.

Liver

The dry matter composition of the livers is shown in figure 4. The DM content of the livers was 24.8%±0.5 in rats fed the 15% protein diet and 24.5%±0.5 in rats fed the 10% protein diet.

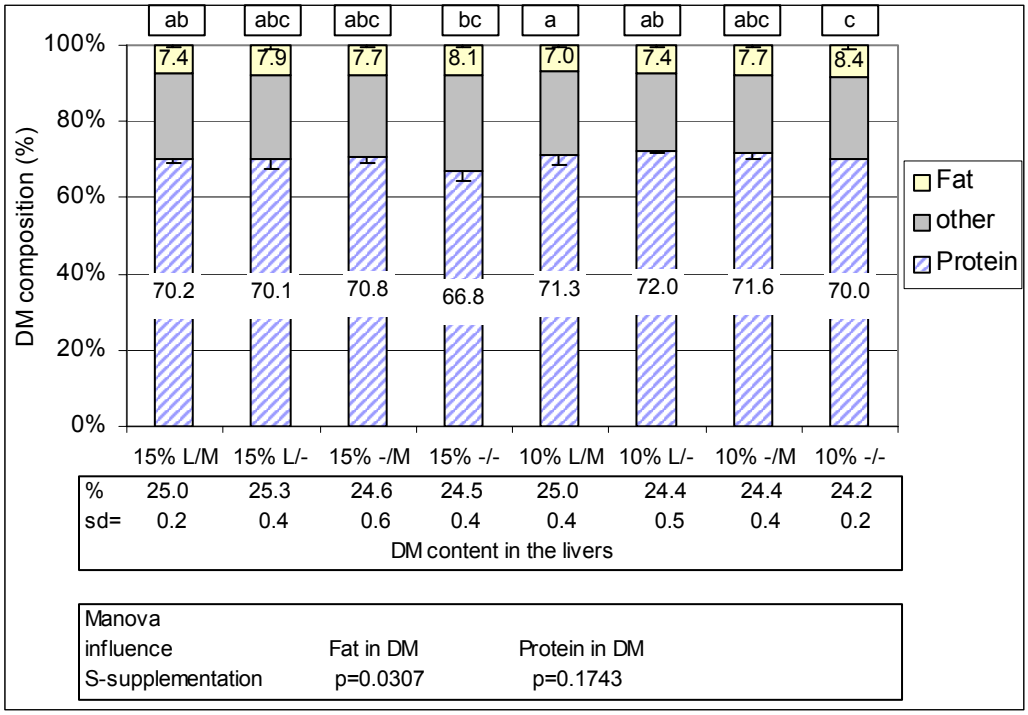

Figure 4. The dry matter (DM) composition and content of the livers of rats after 19 days being fed a 15% or a 10% protein diet deficient in lysine and methionine with no amino acid supplementation or supplemented with free lysine (L) and/or methionine (M). Significant differences between groups ($p<0.05$) are indicated by different letters (only for fat content).

Free amino acid supplementation resulted in a statistically significant effect on the percentage of fat in the DM of the livers. The highest fat content was observed in the groups fed the amino acid deficient diets (15% -/- and 10% -/-) with a mean value of 8.2% ± 1.0. This value differed significantly from the groups fed the complete diets supplemented with the two free amino acids (15% +/+ & 10% +/+) with mean value of 7.2% ±0.6 of DM.
Methionine deficiency resulted in a slight increase in the fat content of the livers independent of the percentage of protein present in the diet (15 or 10%).

DISCUSSION

The aim of the present study was to investigate to which extent deficiency in lysine and methionine affects weight gain and composition of the body and the livers with respect to fat and protein deposition.

We found a positive relation between the lysine content of the diet and weight gain. The more lysine was present in the diet the better growth was observed independent of the protein level of the diet (table 2). Methionine supplementation of the diets with deficient levels of this amino acid did not significantly influence the weight gain of the animals during the experiment. These results could be explained by assuming that despite a deficiency in sulfur containing amino acids, there is still a sufficient amount of methionine and cysteine available in the diet to have normal growth of the animals.

The results of the experimental groups in which methionine was supplemented to the 15% protein diet showed, within the same lysine level, a tendency to reduce the weight gain compared to the animals fed this diet as methionine deficient. This suggests that the presence of methionine limits the availability of lysine to stimulate growth. This could possibly be explained by the methylation of lysine by methionine to carnitine. The diet, which consisted mainly of vegetable components, was poor source of carnitine and, therefore, the rats had to synthesize carnitine themselves from lysine and methionine. The availability of carnitine makes it possible to use fatty acids for energy expenditure. This assumption offers support for the observed tendency of a

decreased fat deposition in the body and livers of rats fed the 15% protein diet supplemented with methionine, on the expense of weight gain.

The involvement of lysine and methionine in carnitine synthesis is further supported by the observation of a significantly higher fat deposition in the liver in case rats were fed a methionine and lysine deficient diet (15% -/- & 10% -/-) compared to the situation when both amino acids were supplemented. In other words, shortage of both amino acids affected carnitine synthesis resulting in increasing fat deposition in the livers.

In the rats fed the 10% protein diet methionine supplementation did not tend to reduce body weight increase within the same level of lysine. Nevertheless, the fat content in the livers of these rats was decreased. This suggests the presence of a lysine independent mechanism in fat utilization (independent of carnitine synthesis). Methionine supplementation could improve the choline status of the body either by favouring choline synthesis or by inhibiting dietary choline from acting as a methyl donor for biological methylations in the body (Sheard et al., 1989). Choline deficiency is known to cause fatty livers. Choline is required for the synthesis of the phosphatidylcholine part of very low density lipoproteins (VLDL). In the absence of choline, VLDL particles cannot be excreted by the liver and triacylglycerol will accumulate in the cytosol of the hepatocytes (Fast et al., 1995, Vance et al., 1971). These results are supported by the observation in the present study that regardless of the protein level in the diet, methionine deficiency caused a slight increase in fat deposition in the liver. These data further suggest that a more general relation can be made between an impaired choline status in the body and methionine deficiency.

A small modulation in weight gain by methionine supplementation of the 15 and 10% protein diets is in line with the fat content in the body. When rats were fed the 15% protein diet, a suppression in weight gain in presence of methionine was accompanied by a decrease in body fat content. Stimulation of

weight gain by methionine supplementation in animals fed the 10% protein diet was accompanied by an increase in the fat content of the body.

CONCLUSIONS

1. Lysine deficiency was limiting for rat weight gain.
2. Methionine deficiency was not limiting for rat weight gain but tended to increase the fat content of the livers.
3. Methionine and lysine deficiency had a synergistic effect on the fat content of the livers.
4. A possible interaction between lysine and methionine supplementation influences body weight gain. When taken into account the fat content of the liver, the results may be explained by the involvement of methionine in liver fat metabolism.

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General Discussion

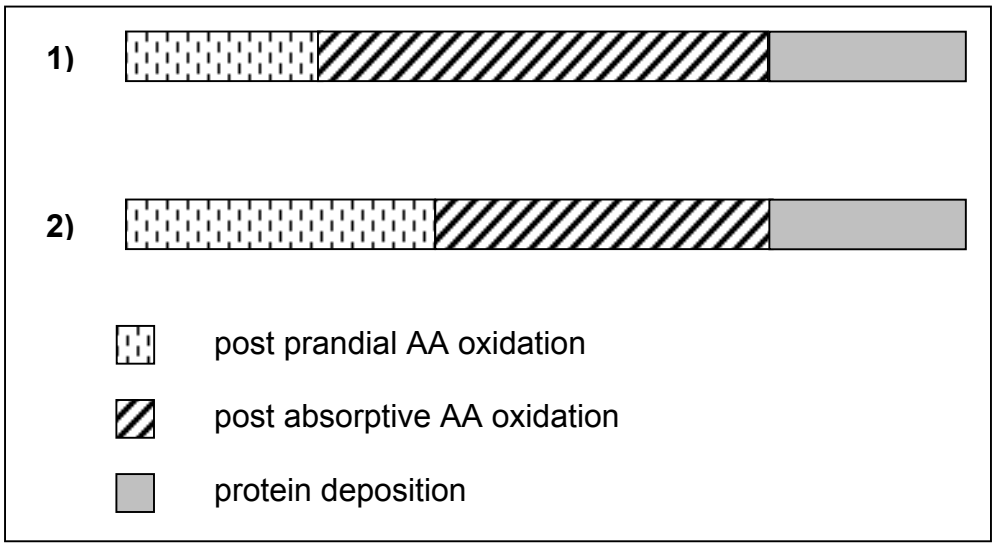
The aim of this dissertation was to study the consequences of different strategies of free amino acid supplementation to dietary protein, on amino acid and protein metabolism, thus determining the consequences of these diets for the physiological utilization of dietary amino acids.

The fate of dietary and endogenous amino acids in relation to the meal composition was studied. Both short and long-term body responses to different nutritional situations were determined. The irreversible short-term catabolic losses of dietary amino acids were studied using the so-called breath test in rats (chapter 1, 2 and 3), an important tool to determine the effects of the amino acid utilization under different nutritional conditions. In chapters 3 and 4 changes in body composition were analyzed with main focus on the fat content of the livers. In all experiments described in this thesis, body weight gain was followed.

Short-term and long-term response to different nutritional situations with regard to amino acids.

Body weight gain is a very simple measurement to follow long-term responses to nutritional situations in both human and animal studies. In this project, through all the experiments weight gain development was carefully monitored. It was expected that different strategies of free amino acid use would influence the body weight. That occurred in animal studies in chapters 1, 3 and 4. In chapter 1, weight gain decreased when free amino acids replaced bound amino acid in the diet. In chapter 3, four different protein levels in the diet and a time interval (0 vs. 1h) of free methionine supplementation influenced weight gain. In chapter 4, the amount of lysine in the diet determined weight gain. However, in the study with 13.7 % protein in the diet that was 50% methionine deficient, weight gain of animals fed with the different time interval (0, 1, 2 or 4 hours) for the free methionine supplementation (chapter 2) did not differ between the interval groups. At the same time, lower post prandial catabolic losses of egg white bound leucine were observed when

free methionine was supplemented with an 1 hour interval. The explanation for this is that apparently post prandial amino acid oxidative losses do not automatically influence growth. Post prandial phase is followed by post absorptive phase and they seem to cooperate. When there are high amino acid oxidative losses in the post prandial phase it is possible that they are compensated by lower oxidative losses in the post absorptive phase. As a result, weight gain is not affected. That holds for adults as well as for growing subjects. It appears that the post absorptive state is very flexible and adjusts to the post prandial state. However, the same long-term outcome (protein deposition) while different short-term (post prandial and post absorptive oxidation) responses does not mean the same physiological status in the two situations as shown in scheme 1. For example in case of leucine, percentage of decarboxylation (the first irreversible step in the degradation of this AA) indicates which part cannot be used for protein synthesis (Weijs, 1993). Therefore, a low oxidative loss in post prandial phase is beneficial for protein status of the body during that period. On the other hand, high oxidation in post absorptive state indicates a high protein turnover. That may be favorable for maintenance processes. This is probably beneficial for the body because more amino acids are mobile and thus available to meet urgent demands as creation of antibodies or acute phase proteins.



Scheme 1. Short-term (post prandial and post absorptive AA oxidation) and long-term (protein deposition) responses of the body to different nutritional situations.

1) low post prandial AA oxidation + high post absorptive AA oxidation = X protein deposition

vs.

2) high post prandial AA oxidation + low post absorptive AA oxidation = X protein deposition

We concluded that the time interval for free methionine supplementation has no long-term effect in terms of utilization for weight gain with methionine deficient protein (13.7%). In this case less methionine is used for other functions than for protein synthesis purposes. In the short-term, an 1 hour interval gave a decrease in post prandial leucine oxidative losses. On the other hand, a high level of post absorptive leucine oxidative losses may indicate a higher protein turnover.

In a subsequent experiment, different protein levels were introduced. We expected that absolute amount of methionine deficiency, in addition to methionine deficient amino acid pattern and time interval, would give a long-term response. Weight differences between 0 and 1h intervals within protein level groups were observed only for 5% and 13.8% protein diets. It appeared that the 1h time interval for the methionine supplementation was beneficial for the 5% protein diet and it had an adverse effect on growth at the 13.8% protein

diet. Moreover, in a study with a diet containing a 7.5% protein level that was 50% methionine deficient, weight gain was similar to the treatment with methionine supplementation at 0 or 1h interval (unpublished data). At that point, we started to wonder if methionine deficiency has any effect on growth. Because of this, the experiment in chapter 4 was designed to compare the influence of methionine and lysine deficiency on weight gain and body composition of growing rats (chapter 4). It showed clearly no influence of a methionine deficiency (34 % below NRC requirements) on growth. However, as expected lysine deficiency (45 % below NRC requirements) came out to be the growth limiting amino acid.

[$^{13}CO_2$]-breath test approach as a method to study short term catabolic losses of dietary and endogenous amino acids

[$^{13}CO_2$]-breath tests are attractive due to the use of non-radioactive substrates and the simple non-invasive sampling technique (Stellaard and Geypens, 1998). That made it widely used in human studies. A [$^{13}CO_2$]-breath test examines the expiration of [$^{13}CO_2$] as function of time after oral intake of a [1-^{13}C]-labeled test substrate (single dose). In clinical settings, breath test studies are often used as a tool to diagnose metabolic functions. From a nutritional point of view, breath test studies can also be used to trace the catabolic fate of dietary nutrients (Schreurs and Krawielitzki, 2003).

In animal studies, the aim is to replace invasive techniques by those which cause less suffering and distress. Keeping that in mind, we also decided for IP route of tracer delivery to study endogenous leucine oxidative losses (chapter 1) influenced by dietary amino acids. The IP injection avoids the need for surgical placement of catheters for IV (intra venues) tracer infusion, in turn eliminating risks of post surgical infection and changes in protein metabolism due to the surgery (Bregendahl et al., 2004; Essen et al., 1992; Tjader et al., 1996). To our knowledge, no published information exists on [$^{13}CO_2$]-breath tests studies after IP administration of the tracer. Nevertheless, the common opinion is that metabolism of ingested or intragastrically administered amino

acid is indicative of what happens to dietary amino acid, whereas metabolism of intraperitoneally administered AA is indicative of liver metabolism and of the metabolism of endogenous AA (Stipanuk and Rotter, 1984).

In our study, we injected 3 mg of Leu to each animal. This amount has been found to be about the threshold value of a tracer dose that does not increase the level of oxidation in itself. At the same time, this amount of a tracer is the minimal amount that is needed for the measurement of the [^{13}C]-enrichment of CO_2 IRMS analyzer (Schreurs and Koopmanschap, 1996).

The tracer dose was the same for IP injection as when ingested with a meal (chapter 1). Such amount caused high oxidation of IP injected tracer within short time, reaching maximal tracer expiration rate (much higher than in case of tracer ingested with a meal) after 30 minutes post injection. In case of IP injection, the tracer by-passed the digestive tract and the whole amount appeared in the free amino acid pool. So it is suppose to enter the endogenous AA pool completely (Schreurs at el, 1992). Previous studies with pigs, chickens and rats showed a rapid distribution of the IP injected tracer (phenylalanine) from the peritoneal cavity to the plasma free pool, reaching maximal tracer enrichment 15 min postinjection (Bregendahl et al., 2004; Martinez, 1987; Czerwinski et al. 1998).

The tracer provided with a meal has to undergo time consuming hydrolyses when bound in egg white and subsequent absorption. In addition, the availability of nitrogen and amino acids varies with protein source and is affected by the presence of antinutritional factors, processing treatments and interaction among other components of the diet (Reeds et al., 2000). Some experiments with isotopes suggest that the luminal digestion of most dietary proteins is virtually complete (de Lange et al. 1990, Gaudichon et al. 1996, Lien et al. 1997, Mahé et al. 1994, Roos et al. 1995). In any case, it has been shown that less than 100% of ingested amino acids after a single meal appear in the portal blood (Stoll et al., 1998; Ebner et al., 1994; Rerat et al., 1988 and 1992). Studies in humans and animals with intragastrically administered, stable

isotope-labeled free amino acids have shown that nutritionally important quantities of essential AA are metabolized in first pass by the tissues of the splanchnic bed (Basile-Filho et al. 1997, Biolo et al. 1992, Hoerr et al. 1991 and 1993, Yu et al. 1992). In addition, it appears that the intestine itself is the dominant organ for the splanchnic utilization of leucine and phenylalanine rather than the liver (Yu et al. 1990 and 1992; Stoll et al. 1997 and 1998). Low portal amino acid appearance (ranging from 40 to 70%) indicates the participation of the gut in modulating the quantity and profile of amino acids delivered to the rest of the body (Shoveller et al., 2005; Baracos, 2004; Stoll et al., 1998; Bertolo et al., 2000).

In the different studies, gradual gastric empting of dietary derived tracer influenced the kinetics of its oxidative losses. Temporary storage in the stomach can prevent sudden appearance of high amount of amino acids at time. It has been recognized for years that the stomach functions as a reservoir that aids in maintaining homeostasis through a gradual emptying of nutrients for digestion and absorption in the small intestine after the mixing of the chyme (Mahe et al., 1992; Read et al., 1982; Low, 1990). This was the reason for prolonged duration of dietary tracer oxidation after a few hours of breath test measurements compared to just about 90 minutes tracer recovery in breath after IP injection.

All factors mentioned above can influence the catabolic fate of amino acids measured with [$^{13}CO_2$]-breath test. In case of leucine, the expiration of [$^{13}CO_2$] reflects the decarboxylated part of the amino acid that is lost from the body. The factors contributing to appearance of dietary derived amino acids are bypassed when amino acid is IP injected. Therefore, we claim that IP injected amino acids face the same fate as body derived (endogenous) amino acids.

The expiration of ingested labeled leucine started in all our breath test studies within the first sampling period, that is within 15 or 30 minutes. The maximal recovery of dietary label was seen after 45 min for free leucine and after 90 min for leucine derived from egg white protein (chapter 1). This indicates very rapid absorption of the labeled exogenous amino acid and

subsequent metabolism. On the other hand, the early expiration of the exogenous tracer might also be from the part of the dietary leucine oxidized by the gut mucosa. In fact, some studies claim a high level of catabolism of dietary essential amino acids in the intestine during first-pass metabolism (Stoll et al., 1998). It may also be that the amino acids are very rapidly absorbed into the blood and catabolised after that. Portal absorption of lysine and threonine was observed already within the first blood sampling period (after 30 minutes from meal ingestion) (Yen et al., 2004).

Short-term (within up to 450 minutes) post prandial catabolic losses of leucine incorporated in dietary protein (egg white) were substantial in our studies. For free leucine oxidative losses (chapter 1) were twice as high as for egg white bound leucine (chapter 1, 2, 3). Moreover, the oxidation of the free amino acid tracer started prior to the oxidation of label derived from protein. Following a single meal, crystalline amino acids appear more quickly in portal blood than amino acids that are ingested as intact proteins or as peptides (Rerat et al., 1992). As a result, crystalline amino acids are more prone to oxidation before they can be used for body protein synthesis than amino acids derived from dietary protein. This is logic because protein synthesis requires that all amino acids are available in the appropriate balance and at the same time (Tuitoek et al., 1997). This implies a lower efficiency of crystalline amino acids for body protein deposition than that of available amino acids derived from intact proteins. This is in line with earlier studies (Matthews, 1975).

From the cumulative recovery values for egg white bound [1-^{13}C]-leucine (chapter 2) we concluded that diets with methionine deficient protein and free methionine given simultaneously do not have the lowest oxidative losses. The best result was obtained when the methionine was given one hour after the methionine deficient meal. In another study, we found that the oxidative losses of egg white bound [1-^{13}C]-leucine in animals with 0 and 1h interval of free methionine supplementation were not significantly different (chapter 3). The discrepancy between the results of the two studies indicates the importance of

age, diet, feeding pattern, etc. in the process of gastric emptying, digestion, absorption and oxidation of amino acids. Apparently, those physiological factors were not identical. The IP injected tracer's oxidative response in the breath was visible only up to about 90 minutes after injection with maximal response already after 30 minutes. This is because of short half-life of amino acids in the free endogenous AA pool. Actual amounts of free amino acids in the blood are sufficient to support whole body protein synthesis for some minutes only (Schreurs et al., 1997). Therefore, it is important to synchronize the time of labeling the endogenous pool with the time when dietary derived amino acids appear in the endogenous pool. In other words, we assume that if IP injection of labeled leucine is postponed after ingestion of dietary amino acids the different nutritional situations will give more differences in the breath test results. Nevertheless, it was shown in our study (chapter 1) that the expiration of the endogenous leucine was modulated by the dietary amino acids. In the presence of dietary derived leucine, oxidative losses of endogenous leucine increased. In case of the presence of dietary derived amino acids without leucine, oxidative losses of endogenous leucine decreased. This implies that at the site of oxidation the body cannot distinguish between exogenous and endogenous AA both for oxidation and for deposition. It has to be stressed that this does not apply to the gut. After ingestion, dietary proteins are mixed with endogenous proteins secreted in the lumen, e.g., gastric, biliopancreatic and intestinal secretions, and sloughed epithelial cells (Alpers, 1994; Matthews, 1990). Endogenous protein is generally accepted to be digested at slower rates than dietary protein (Matthews, 1990).

In summary, the [$^{13}CO_2$]-breath test can be used to study short-term catabolism of dietary and endogenous amino acids in different nutritional situations (chapters 1, 2 and 3). It can be considered as an indicator for short-term response of the body to amino acid supply. The effect on the long term (weight gain and body composition) does not need to be the same. Kinetics of label recovery in breath test study provides interesting data for physiological explanation (as discussed below with methionine).

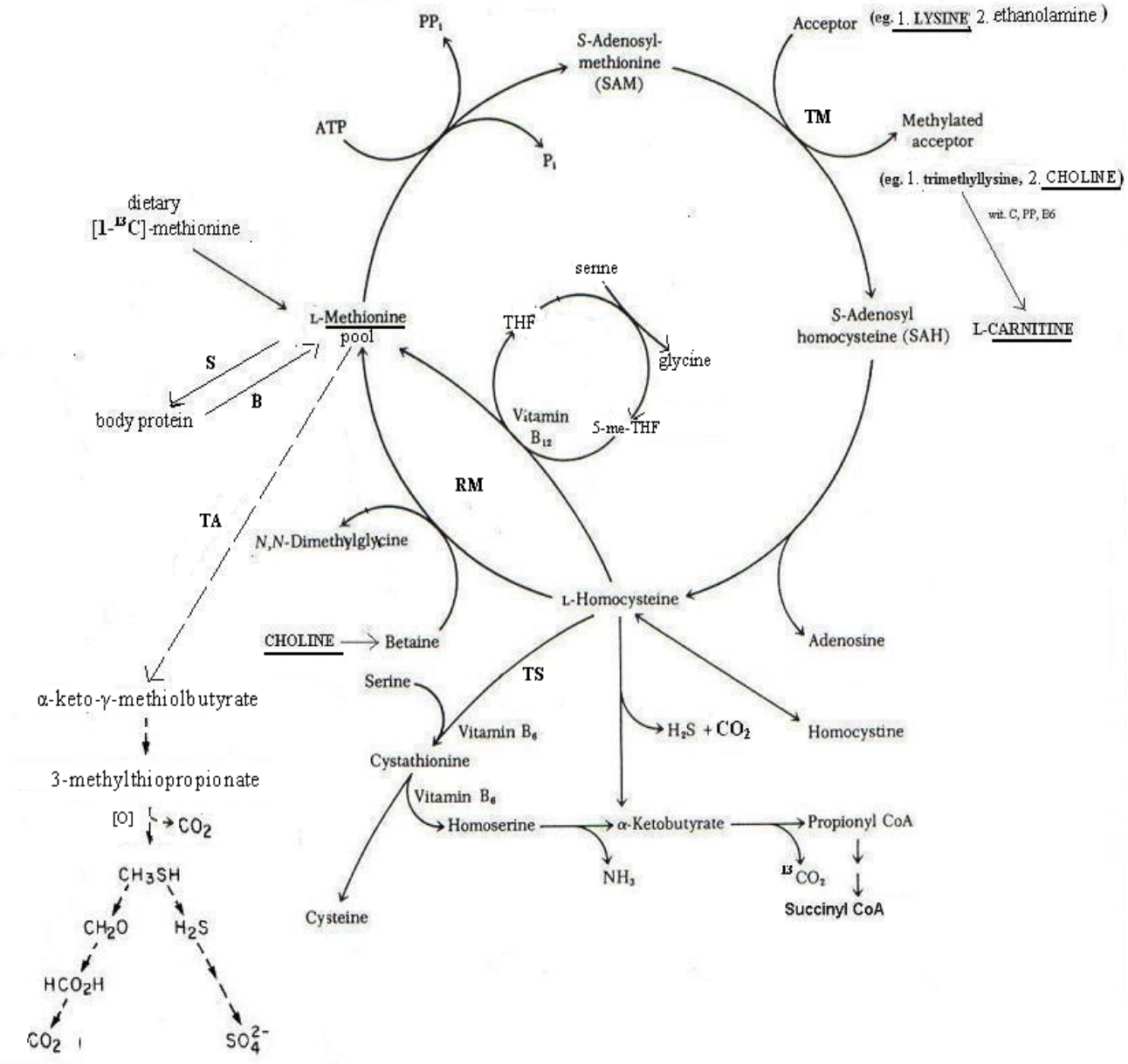
Physiological consequences of dietary methionine deficiency for growing rats.

Methionine is an essential amino acid mostly metabolized by the liver (Milazzo et al., 2005). It can either be incorporated into proteins by protein synthesis (S) or undergo transmethylation (TM). After that the methyl group is transferred to a methyl acceptor. Through the intermediate *S*-adenosylmethionine (SAM), methionine is the source of the methyl groups of choline, creatine, carnitine and both DNA and RNA intermediates (Aguilar et al., 1974; Cooper, 1983; Storch et al., 1988; Griffith, 1987; Stipanuk, 1986). Transmethylation yields *S*-adenosylhomocysteine, which is hydrolyzed to form homocysteine. The last one can be remethylated (RM) to methionine by one of two pathways (scheme 2) or undergo transsulfuration (TS) (MacCoss et al., 2001). Remethylation back to methionine takes place via accepting a methyl group from 5-methyl-tetrahydrofolate (5-me-THF) or betaine (metabolite of choline) (Fukagawa et al., 2000; Niculescu and Zeisel, 2002). The transsulfuration pathway is the major route of methionine catabolism (Finkelstein and Mudd, 1967). It starts with condensation of homocysteine with serine to form cystathionine. Cystathionine is then reduced to form cysteine, with the C-backbone of the original homocysteine being oxidized and only the sulfur being transferred to the resulting cysteine (MacCoss et al., 2001).

Scheme 2 shows pathways for methionine metabolism.

In vivo animal studies have shown transamination to α-keto-γ-methiolbutyric acid (dotted line in the scheme 2) as a possible route for methionine degradation (Bloom, 1989; Gahl et al, 1988; Mitchell and Benevenga, 1978). In particular, patients with hypermethioninemia, due to a defect in the transsulfuration pathway, may catabolize significant amounts of methionine via the transamination pathway (Bloom, 1989; Gahl et al, 1988). Otherwise, in physiological situations this transamination quantitatively is not an important route for methionine degradation and therefore negligible (Bloom et

al., 1989; Cooper, 1989; Wu and Thompson, 1989; Stipanuk, 1986; Finkelstein and Martin, 1984).



Scheme 2. Methionine metabolism. Interaction between methionine, lysine, carnitine and choline. (S-synthesis, B-breakdown, TM-transmethylation, RM-remethylation, TS-transsulfuration, TA-transamination)

In summary, methionine metabolism is a complex process. To simplify this, Finkelstein (1998a, 1998b, 2000) suggested to consider in mammalian tissues 2 pathways for methionine metabolism: the methionine cycle and transsulfuration. Only the liver in humans contains both pathways of methinone

metabolism in complete form (Finkelstein, 1998b). Every tissue possesses the methionine cycle and thus, all cells can conserve methionine and recycle folates. Transsulfuration is restricted in the rat to liver, kidney, small intestine, and pancreas. Methionine is a source of cysteine only in these tissues (Finkelstein, 1988). Both the rate of cycling as well as the distribution of homocysteine between the two routes are the basis for metabolic regulation and can be separated in time and space (Finkelstein, 1990).

In this project, short-term catabolic losses of free dietary methionine were followed after it supplementation with 0, 1, 2 and 4h interval after the deficient protein (13.7%) meal (chapter 2). In chapter 3, catabolism of free methionine was measured when supplied either after a 0 or a 1h interval after the 50% methionine deficient protein meal (5% protein). It was noticed in both studies that in spite of comparable requirements recommended by National Research Council (1995) for growing rats for methionine + cysteine (9.8 g/kg of feed) and leucine (10.7 g/kg of feed), methionine oxidative losses were much higher than leucine. In contrast to leucine, the high oxidative losses of methionine should not always be considered as complete losses. It has to be remembered that the irreversible transsulfuration pathway of homocysteine to form cysteine yields CO_2 that is measured using breath test technique. This means that part of the methionine molecule can still be used as methyl donor and sulfur donor for cysteine synthesis (see scheme 2).

In chapter 3, an interesting pattern of the label recovery from free [1-^{13}C]-methionine was observed for the 1h interval group fed 5% protein diet. The first carbon (C_1) labeled in the tracer (as used in the present project) is conserved with homocysteine conversion back to methionine (scheme 2). In other words, the labeled methionine after first transmethylation can then be remethylated. This temporal conservation might be responsible for the biphasic kinetics of labeled methionine recovery. Earlier studies suggest that methionine conservation is achieved by an increase in homocysteine remethylation relative to transsulfuration via cystathionine synthesis. On a low protein diet (3.5%

casein) the need to conserve methionine was met by an increase in homocysteine remethylation and a decrease in transsulfuration (Finkelstein and Martin, 1984). Studies of the effects of variations in the dietary content of methyl groups in humans also indicated that the percentage of homocysteine remethylated could vary from 70% during periods of restricted intake to approximately 40% when the amount of methionine and choline was increased (Mudd and Poole, 1975; Mudd 1980). Therefore, the biphasic pattern of 1h interval on very low protein diet (5%) in our study (chapter 3) might be explained as a mechanism to cope better with the nutritional situation than in 0h interval group. Even if on short-term the cumulative oxidative losses of the tracer in both cases were the same, long-term 1h interval paid off with better weight gain. This suggests the usefulness of analyzing the kinetics of label recovery in breath test as indicator of physiological consequences.

In our breath test measurements, where [1-^{13}C]-methionine was used, we concluded what part of the labeled methionine was not used directly for protein synthesis. At the same time, one may speculate about the oxidized label that in fact could be used for other purposes. First, methyl group from methionine can be provided and used for choline, creatine, carnitine and both DNA and RNA intermediates. Secondly, sulfur in methionine was used for cysteine synthesis. Which can then possibly be used for body protein synthesis. Therefore, the transsulfuration of methionine catabolic pathway is beneficial for cysteine synthesis but the later can be also catabolized. In fact, transsulfuration is the pathway to catabolize excessive methionine (Finkelstein and Mudd, 1967).

Cumulative recovery of labeled methionine (chapter 2) gave much higher values than for leucine. In case, where there were lowest egg white bound [1-^{13}C]-leucine oxidative losses, there was highest [1-^{13}C]-methionine recovery. That can be explained by higher demand for cysteine from methionine when high dietary protein utilization was observed.

Methionine impact on livers composition

Methionine had an interesting influence on the liver composition. In chapter 3 we found that there is an influence of protein level in the diet on fat content in the livers. On a very low protein level (5%) in the diet fatty livers occurred. It is reasonable to assume that in such nutritional situation methionine deficiency occurred, leading to impairment of the non-protein functions of methionine. Methionine deficiency could impair choline status of the body either by neglecting choline synthesis or by compelling dietary choline to act as methyl donor for biological methylations (Zeisel 1991). Choline is known to be required for the phosphatidylcholine part of very low-density lipoproteins. In the absence of choline, VLDL particles cannot be excreted from the liver and triacylglycerol accumulates in the hepatic cytosol. Therefore, choline deficiency is known to cause fatty livers (Fast et al. 1995, Vence, 1991). In chapter 4, we did not apply such extreme low protein diets. This can explain why we found no influence of the protein level itself in the diet (10% vs. 15%) on fat content of the liver. Instead, supplementation with methionine and lysine had a significant impact on fat content in the livers. We found out that both lysine and methionine deficiency caused more fat deposition in the livers compared to situation when both amino acids were supplemented. The increase in fat deposition when there is lysine and methionine deficiency could be caused via affecting the carnitine status in the body. Lysine is methylated by methionine to carnitine. In case of shortage of both amino acids required for carnitine synthesis, use of fatty acids for energy expenditure was affected. Carnitine enables fatty acids to be used as a source of energy via β-oxidation (Hoppel C.L. 1998).

Protein level in the diet

In this project, we assumed that an animal with a low protein level in the diet would be more sensitive to different strategies of free methionine supplementation. Although influence of protein level in the diet on body

composition was not a main interest for us, we want to mention the consistency or some discrepancies of our study with the literature.

On high dietary protein level (13.8%), there was more body protein and less body fat content in DM compared to animals on the 5% protein diet (chapter 3). This confirms earlier findings of an increased body fat content in the rats fed low protein diets (Meyer 1958, Noblet et al. 1987, Russell et al. 1983, Swick and Gribskov 1983, White et al. 1994 and 1998, Du et al. 2000). Also in farm animals this has been found (Deschepper and De Groote, 1995; Seve et al., 1986)

In chapter 4, more fat deposition on the 15 % protein level was found compared to the 10% protein level diet. This can be explained by the higher energy content. The protein level was probably not limiting in that study. There was a big drop in feed intake in the study reported in chapter 3 caused by anorexia on the 5% protein diet. Because of that, the maximum feed intake in this experiment was 12g a day. Whereas in experiment in chapter 4, the feed intake was gradually increased up to 17.5g a day. In other words, excessive energy supply with intake of 17.5 g of feed a day caused extra fat deposition on 15% protein diet. The extra dietary protein was not needed for protein deposition; therefore, it was channeled to fat deposition. It was even more pronounced when methionine was not supplemented on 15% protein diet. It seems like shortage of methionine at the same level of lysine affected carnitine synthesis and therefore more fat deposition occurred on the weight gain expense. This pattern is not seen on 10% protein diet probably due to absolute amount of methionine differences.

GENERAL CONCLUSIONS

In this dissertation, consequences of different strategies of free amino acid supplementation to dietary proteins for physiological utilization in growing rats were studied. From the results, the following was concluded:

► [$^{13}CO_2$]-breath test technique was a useful tool to study short-term catabolic losses of dietary and endogenous (IP injected) amino acids.

► Short-term catabolism of dietary free leucine considerably exceeded catabolism of egg white bound leucine.

► Short-term catabolism of endogenous leucine was modulated by exogenous amino acid supply and increased or decreased depending on the adequacy of the dietary supply.

► Post prandial catabolism of dietary methionine was considerably higher than that of leucine, indicating that only a small amount of dietary methionine compared to leucine was deposited in body protein.

► Time interval for free methionine supplementation influenced post prandial oxidative losses of egg white bound leucine and free methionine. Therefore, introducing time interval of free amino acid supplementation to poor quality protein can be considered as a factor with potential to improve physiological utilization of dietary amino acids.

► Post prandial amino acid oxidative losses not always influenced growth but probably influenced post absorptive amino acid catabolism (indicator of protein turnover).

► 34% methionine deficiency below NRC recommended amino acid pattern was not limiting for rats' growth but influenced fat metabolism probably via choline and carnitine.

► Lysine deficiency limited rat's weight gain.

► Methionine and lysine deficiency caused significantly higher fat deposition in the livers compared to situation when both amino acids are supplemented.

RECOMMENDATIONS FOR FUTURE STUDIES

After completion of this dissertation, a number of areas remain open for further research. Following topics can be of interest:

► Further studies on different strategies of supplementation for various free amino acids aiming to lower post prandial amino acid losses eg. lysine as probably more relevant for short and long term protein deposition.

► Studies on meaning of variation in post prandial metabolism of amino acids for adaptation to stress.

► Studies on differences in the outcome of strategies of free amino acid supplementation between various species and physiological conditions.

► Studies aimed to evaluate feedstuffs, which are best used for specific supplementation strategies.

► Study to estimate net methionine post prandial oxidative losses aimed to distinguish from methionine catabolism for cysteine synthesis.

► Study to optimize free amino acid supplementation in clinical nutrition in diseases such as phenylketonuria (PKU), maple syrup urine disease (MSUD), chronic renal failure (CRF), etc.

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Summary

Free amino acid supplementation aimed to meet tabulated amino acid requirements is widely used in animal production and clinical nutrition. However, the efficiency of using crystalline amino acids can be lower than that of amino acids derived from dietary proteins. Following a single meal, dietary free amino acids appear in portal blood prior to amino acids derived from the dietary protein. Consequently, part of the crystalline amino acids are subject to early oxidation before they can be used for body protein synthesis in combination with the protein derived amino acids. Therefore, the time lag in absorption between free and protein bound amino acids seems to make a difference in amino acid metabolism and in physiological utilization.

The studies in this thesis were mainly focused on differences in short-term catabolism of protein bound and free amino acids during the post prandial phase of a meal. Stable isotope technique was used in model studies with laboratory Wistar (WU) rats. In different experiments, so-called [$^{13}CO_2$]-breath test studies were used to compare the metabolic fate of free and protein-bound [1-^{13}C]-labeled amino acids in a meal in various nutritional situations.

The aim of the project was to get a better view on dietary conditions that might improve the use of crystalline amino acids in animal diets or in clinical nutrition under practical conditions.

In **Chapter 1**, we showed that short-term catabolism of endogenous leucine is modulated by exogenous amino acid supply and increases or decreases depending on the adequacy of the dietary supply. Lower weight gain and higher post prandial catabolic losses of the tracer were observed when the dietary protein was replaced with the crystalline amino acids.

In **Chapter 2**, it was investigated whether the time interval for the free methionine supplementation of 50% methionine deficient dietary protein improved post prandial amino acid utilization measured in breath tests with egg white bound [1-^{13}C]-leucine and free [1-^{13}C]-methionine. We found that the best utilization of the methionine deficient protein, seen in post prandial oxidative losses of the egg white bound leucine, did not occur when methionine

supplement was given simultaneously with methionine deficient meal. Optimal post prandial nutritional situation for protein bound leucine coincided with higher label recovery from [1-^{13}C]-methionine, probably due to conversion to cysteine.

In **Chapter 3**, the influence of a time interval (0 vs. 1 hour) for the free methionine supplementation of 50% methionine deficient dietary proteins on the short-term post prandial amino acid oxidation and the body composition on diets with different level of the dietary protein (5, 7.5, 10 & 13.8%) was investigated.

The length of the time interval modulated the post prandial catabolic losses of the tracer. The labeled methionine oxidative losses were considerably higher than that of leucine, indicating that less methionine compared to leucine was deposited in body protein. The 1h time interval for the free methionine supplementation influenced the weight gain but differently depending on the dietary protein levels. Animals fed the very deficient protein diet (5%) showed higher weight gain when supplementation of the deficient free methionine was provided with an 1 hour interval. Dietary protein deficiency in this study showed fatty livers when it reached less than 7.5%.

In **Chapter 4**, we studied the impact of lysine and/or methionine deficiency on body weight gain, on the carcass and on the liver composition of growing rats. Lysine deficiency was limiting for rats weight gain. Methionine deficiency was not limiting for weight gain but tended to increase the fat content of the livers. Methionine and lysine deficiency had a synergistic effect on the fat content of the livers. Body weight gain showed a tendency for interaction between supplementation of lysine and methionine. In combination with fat content of the livers, results can be explained by the involvement of methionine in liver fat metabolism.

Overall, we concluded that:

- ► The [$^{13}CO_2$]-breath test technique was a useful tool to study the short-term catabolic losses of the dietary and the endogenous (IP injected) amino acids.
- ► Short-term catabolism of the dietary free leucine exceeded catabolism of the egg white bound leucine.
- ► Short-term catabolism of the endogenous leucine was sensitive for modulation by the exogenous amino acid supply and increased or decreases depending on the adequacy of the dietary supply.
- ► Dietary methionine post prandial catabolism was considerably higher than that of leucine, indicating that less methionine compared to leucine was deposited in the body protein.
- ► Time interval of free methionine supplementation influenced the post prandial oxidative losses of egg white bound leucine and free methionine. Therefore, introducing a time interval of the free amino acid supplementation to poor quality protein can be considered as a factor with the potential to improve the physiological utilization of the dietary amino acids.
- ► Post prandial amino acid oxidative losses not always influence the growth but probably influence the post absorptive amino acid catabolism.
- ► Although lysine deficiency was limiting for rat growth, even a 34% methionine deficiency below the NRC recommendation was not. The latter clearly influenced the fat metabolism most probably via choline and carnitine.

Samenvatting

Het gebruik van vrije aminozuren als supplement in de voeding vindt momenteel ruime toepassing in de voeding van mens en dier. Het doel van deze toepassing is het bereiken van een optimale afstemming tussen het aanbod van aminozuren in de voeding en de behoefte aan aminozuren van het lichaam. Op het niveau van de darm zijn vrije aminozuren eerder beschikbaar voor resorptie dan aminozuren die eerst uit eiwit moeten worden vrijgemaakt. Hierdoor worden vrije aminozuren niet alleen eerder geresorbeerd maar vertonen zij ook een hogere verschijningssnelheid in het portale bloed. Een verschil in tijdstip en snelheid van absorptie leidt tot een verschil in metabole response. Hierdoor leiden vrije aminozuren niet automatisch tot dezelfde fysiologische benutting als aminozuren die als eiwit in de voeding aanwezig zijn.

Dit proefschrift richt zich voornamelijk op de verschillen in postprandiaal catabolisme tussen aminozuren die in vrije vorm of als eiwit via de voeding worden aangeboden. In modelstudies met Wistar ratten is gebruik gemaakt van [$^{13}CO_2$]-ademtesten. In deze testen wordt een [1-^{13}C]-gelabelde aminozuur, in vrije vorm of ingebouwd in ei-eiwit éénmalig, meestal via een maaltijd, als substraat aangeboden Het verschijnen van [$^{13}CO_2$] in de uitgeademde lucht geeft informatie over het verloop van de decarboxylatie van het [1-^{13}C]-gelabelde aminozuur dat als substraat werd aangeboden.

Het doel van dit proefschrift was om een beter inzicht te krijgen in mogelijkheden om de postprandiale benutting van vrije aminozuren in de voeding van mens en dier te optimaliseren.

In **hoofdstuk 1** werd aangetoond dat zowel het patroon als de hoeveelheid van de aminozuren die via de voeding worden aangeboden een modulerende invloed hebben op het catabolisme van de (endogene) aminozuren die al tijdens de maaltijd in het lichaam aanwezig zijn. Deficiënties in het patroon van de aminozuren die via de voeding werden aangeboden veroorzaakten een verlaging van de oxidatie van de endogene aminozuren. Hieruit werd geconcludeerd dat ook endogene aminozuren een rol spelen bij de postprandiale benutting van exogene aminozuren, die met de voeding worden aangeboden. Deze

wisselwerking tussen endogene en exogene aminozuren kan een positieve invloed hebben op de postprandiale opslag van de aminozuren uit de voeding. Wanneer de exogene aminozuren niet als eiwit maar in vrije in vrije vorm werden aangeboden werd een hogere postprandiale oxidatie en een lagere gewichtstoename gevonden.

In **hoofdstuk 2** werd onderzocht of het aanbrengen van een tijdsinterval tussen het verstrekken van een maaltijd waarvan de eiwitfractie deficiënt is in methionine en het verstrekken van een supplement met vrij methionine invloed had op het postprandiale metabolisme van de aminozuren uit de voeding. De postprandiale oxidatie van de eiwitfractie was minimaal wanneer de deficiënte maaltijd en het supplement met methionine werden verstrekt met een tijdsinterval van 1 uur. In deze studie was de decarboxylatie van het methionine in het supplement verrassend hoog. Dit wijst waarschijnlijk op het verbruik van methionine voor de vorming van cysteine dat nodig is voor de netto eiwitsynthese. Uit deze experimenten werd afgeleid dat het gelijktijdig aanbieden of opmengen van deficiënt eiwit en het supplement metabool gezien niet tot een optimale situatie leidt.

In **hoofdstuk 3** werd onderzocht of de invloed van een tijdsinterval tussen het verstrekken van een deficiënte maaltijd en een supplement met methionine op het postprandiale metabolisme van de aminozuren uit de voeding afhankelijk was van het eiwitniveau van het dieet (5, 7.5, 10 & 13.8%). Het tijdsinterval had een modulerende effect op het postprandiale verlies van aminozuren. Ook in deze studie werd in alle gevallen voor het methionine in het supplement een aanzienlijk hogere decarboxylatie gemeten dan voor leucine uit de eiwitfractie. Dit geeft aan dat aanzienlijk minder methionine dan leucine in eiwit werd ingebouwd. Alleen voor het 5% eiwitniveau had het verstrekken van het methionine supplement na 1 uur ook een positief effect op de gewichtsontwikkeling van de dieren. Bij een eiwitniveau lager dan 7.5 % werd een sterke toename in vervetting van de lever gevonden.

In **hoofdstuk 4** werd een vergelijking gemaakt tussen de invloed van een deficiëntie van lysine en methionine op de ontwikkeling van het lichaamsgewicht

en de samenstelling van het karkas en de lever. Het gehalte aan lysine in de voeding was duidelijk limiterend voor de gewichtsontwikkeling. Het gehalte aan methionine in de voeding had daarentegen nauwelijks invloed op het gewicht, maar wel een duidelijk effect op het vetgehalte van de lever. Voor zowel de ontwikkeling van het lichaamsgewicht als het vetgehalte van de lever werden interacties gevonden tussen het gehalte van lysine en methionine in het voer. De resultaten wijzen op een belangrijke rol voor methionine uit de voeding bij het voorkomen van vetstapeling in de lever.

Samenvattend heeft dit proefschrift tot de volgende conclusies geleid:

► De [$^{13}CO_2$]-ademtest kan worden gebruikt voor het bestuderen van de metabole afbraak van zowel exogene als endogene aminozuren direct na een maaltijd.

► Zowel het patroon als de hoeveelheid van de aminozuren die via de voeding worden aangeboden hebben een modulerende invloed op het catabolisme van de endogene aminozuren.

► De metabole afbraak van aminozuren uit de voeding is doorgaans verhoogd wanneer de aminozuren niet als eiwit maar in vrije vorm in de voeding aanwezig zijn.

► Bij een voeding, samengesteld conform de aanbevelingen voor aminozuren, is de postprandiale afbraak van methionine veel hoger dan van leucine. Dit betekent dat methionine relatief minder wordt ingebouwd in eiwit en kennelijk andere functies vervult.

► Het gelijktijdig aanbieden of opmengen van een methionine deficiënt eiwit en het methionine supplement leidt, metabool gezien, niet tot de meest optimale situatie. Het toepassen van interval supplementatie verdient overweging voor practische toepassingen.

► De postprandiale verliezen van aminozuren hebben niet altijd invloed op de groei maar hebben wel invloed op het postabsorptieve metabolisme (turnover) van de aminozuren uit de voeding.

► In tegenstelling tot lysine is een deficiëntie aan methionine nauwelijks limiterend voor de gewichtsontwikkeling van ratten. Methionine voorkomt, waarschijnlijk via choline en carnitine, een stapeling van vet in de lever.

Appendixes

Acknowledgement

List of Publications

Training and Supervision Plan

About the Author

Acknowledgments

This thesis is the product of a long cooperation between my supervisors: prof. Martin Verstegen and dr. Victor Schreurs from the Wageningen Institute of Animal Sciences at Wageningen University and dr. Jacek Bujko from Faculty of Human Nutrition and Consumer Sciences at Warsaw Agricultural University (WAU). Being a PhD student at the WAU with dr. Bujko as my first contact supervisor, I was introduced to Victor Schreurs during the WIAS course on "Stable Isotopes in studies of Nutrient Dynamics" in 2001. The next year I was invited to Wageningen for a 3-month fellowship. The following years I spent a few months a year doing my research in Wageningen and going back to Warsaw to follow the PhD program there. The Polish-Dutch cooperation got stronger with every following experiment, course, conference and other events involving both sides. In 2004, I decided to graduate from Wageningen University. On top of the scientific support, I had great assistance from my supervisors in overcoming the administrative difficulties that came with the decision of the transfer.

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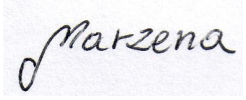

List of Publications

In preparation for submission

Gas M, Verstegen MWA, Bujko J, Renkens M, *Koopmanschap RE and Schreurs VVAM:* Short term catabolism of endogenous amino acids in rats is enhanced by exogenous amino acids supply. [$^{13}CO_2$]-breath test study with L-[1-^{13}C]-leucine.

Gas M, Bujko J, Krzyzanowska M, *Koopmanschap RE, Verstegen MWA and Schreurs VVAM:* A 1h time interval for free methionine supplementation reduces post prandial amino acid oxidative losses of methionine deficient dietary protein.

Gas M, Bujko J, Chudobinska E, *Koopmanschap RE, Verstegen MWA and Schreurs VVAM:* Influence of free methionine supplementation interval on post prandial amino acid catabolism and body composition at different levels of protein intake.

Gas M, Bujko J, Krupa K, *Koopmanschap RE, Verstegen MWA and Schreurs VVAM:* Impact of lysine and/or methionine deficiency on body weight gain and on body and liver composition in growing rats.

Gas M, Bujko J, Banasiuk K, *Koopmanschap RE, Verstegen MWA and Schreurs VVAM:* Postprandial [1-^{13}C]-leucine short term catabolism in rats fed lysine deficient diets supplemented with free lysine after different time intervals.

Conference papers

Gas M., Myszkowska-Ryciak J., Bujko J., Schreurs V.V.A.M., Koopmanschap R.E.: "Influence of meal frequency and dietary protein source on post absorptive leucine oxidation in rats" Human Nutrition and Metabolism, Warsaw, April 2003, 1224-1228. (article in Polish)

Bujko J., Gas M. Krzyżanowska M., Koopmanschap R. E., Schreurs V.V.A.M.: „Optimal time interval for amino acid supplementation as studied by amino acid oxidation during the postprandial phase". In: Progress in research on energy and protein metabolism (Souffrant WB. & Metges CC., eds.), EAAP publication No. 109, 2003, Rostock-Wernemunde, Germany 13-18.09.2003.

Bujko J., Myszkowska-Ryciak J., Gas M., Groberek J., Koopmanschap R.E., Schreurs V.V.A.M.: "The influence of meal size on postprandial leucine oxidative losses in humans" in: Selected problems in the Science of Human Nutrition at the beginning of XXI century, Warsaw Agricultural University Press, 2004, 79-82. (article in Polish)

Gas M., Bujko J., Krupa K., Koopmanschap R.E., Schreurs V.V.A.M.: „Impact of Lys and/or Met deficient protein on body weight gain and carcass composition of growing rats." In: Conference material during "Physiological Determinants of Progress in Diet Therapy", Warsaw Agricultural University Press, 2004, 132-137.

Training and Supervision Plan		**Graduate School WIAS**	
Name	Marzena Gas		
Group	Human and Animal Physiology		
Daily supervisor(s)	V.V.A.M. Schreurs, J. Bujko		
Supervisor(s)	M.W.A. Verstegen		
Project term	from 2001 until 2005		
EDUCATION AND TRAINING (minimum 21 cp, maximum 42 cp)			
The Basic Package		year	cp
WIAS Introduction Course (mandatory)		2005	1.0
Course on philosophy of science and/or ethics (mandatory)		2005	1.0
SUBTOTAL BASIC PACKAGE			2.0
Scientific Exposure		year	cp
International conferences			
"Food and Hygiene of Nutrition a base for health and food safety – a challenge for XXI century", Rogowo, Poland		2003	0.6
"Symposium on Energy and Protein Metabolism and Nutrition", Rostock-Wernemunde, Germany		2003	1.2
"Physiological Determinants of Progress in Diet Therapy", Warsaw, Poland		2004	0.4
Seminars and workshops			
WIAS Seminar plus and workshop "Stable Isotopes in studies of Nutrient Dynamics, Stress and metabolic adaptation", Wageningen		2001	0.3
WIAS Seminar Plus "Dietary protein: Physiological constraints to nutritive value", Wageningen		2004	0.6
Presentations			
"Influence of feeding frequency and protein source on postabsorptive leucine oxidative losses in rats" Rogowo, Poland (poster)		2003	0.5
"Optimal time interval for amino acid supplementation as studied by amino acid oxidation during the postprandial phase" Rostock, Germany (poster)		2003	0.5
"Impact of lysine and/or methionine deficient diets on body weight gain and composition of liver and carcass of growing rats." Warsaw, Poland (poster)		2004	0.5
"Model studies on amino acid supplementation" WIAS Seminar Plus, Wageningen, The Netherlands (oral)		2004	0.5
SUBTOTAL INTERNATIONAL EXPOSURE			5.1
In-Depth Studies		year	cp
WIAS course "Stable Isotopes in studies of Nutrient Dynamics", Wageningen		2001	0.6
VLAG-WIAS course "Ecophysiology of the GI-tract", Wageningen		2005	1.0
"Statistical methods in experiments" Warsaw, Poland (30h)		2003	0.8
"Human Nutrition, selected parts" Warsaw, Poland (45h)		2003	1.0
"New technologies and food standardization" Warsaw, Poland (45h)		2003	1.0
SUBTOTAL IN-DEPTH STUDIES			4.4
Professional Skills Support Courses (minimum 2 cp)		year	cp
Course on Laboratory Animal Science		2005	3.0
SUBTOTAL PROFESSIONAL SKILLS SUPPORT COURSES			3.0
Didactic Skills Training		year	cp
Classes of Dietetics and Human Anatomy with students at Warsaw Agricultural University (total 460 hours including preparations)		2002-2005	11.5
Supervising 4 MSc Students		2002-2004	4.0
SUBTOTAL DIDACTIC SKILLS TRAINING			15.5
Education and Training Total			**30.0**
One credit point (cp) equals a study load of approximately 40 hours			

About the Author

Marzena Gas was born on the 26th of October 1972 in Piaseczno, Poland. In 1992 she graduated from the Agricultural High School in Warsaw, with a specialization in horticulture. The same year she started to study at Warsaw Agricultural University at the Faculty of Human Nutrition and Home Economics. She graduated in 1997 with a master's degree in Food Technology and Human Nutrition. From 2001 she continued her study on the PhD level at the Warsaw Agricultural University. During her doctoral studies she was granted the Marie Currie Fellowship at Wageningen University (3 times for a total of 1 year). During the time spent at WU she carried out all her experiments for the PhD dissertation, which led to the accomplishment of the PhD in 2006.

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