Postprandial fate of amino acids: adaptation to molecular forms

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Postprandial fate of amino acids: adaptation to molecular forms

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Abstract

During the postprandial phase dietary proteins are digested to peptides and amino acids and absorbed. Once absorbed the peptides are further hydrolyzed to amino acids and transported to the tissues. These amino acids are largely incorporated into body proteins. Not all amino acids are, however, incorporated into body proteins part of these amino acids are oxidized, and can, thus, no longer be utilized to support protein metabolism in the body. The objective of this thesis was to increase the understanding of those processes that determine the utilization efficiency of dietary proteins. The studies described in this thesis, focused on the appearance rate of dietary amino acids in the free amino acid pool of the body. The rate of appearance of dietary amino acids in this pool has been shown to modulate the postprandial oxidation of amino acids and thereby also their utilization efficiency for physiological purposes. As a consequence postprandial oxidative losses influence the nutritional protein status of the body. This thesis aimed to elucidate whether the body is able to cope with diets in which the amino acid appearance rate is high and what mechanisms are involved in this process.

First an effort was made to establish the metabolic consequences of amino acid sources with a high appearance rate in a rat and a human model. Postprandial oxidation of free or protein derived [1-^{13}C]-leucine was determined in a [$^{13}CO_2$]-breath test, using both a diet consisting of only free amino acids including [1-^{13}C]-leucine and a diet consisting of proteins in which [1-^{13}C]-leucine was incorporated, and 1:1 mixtures of both diets. In those mixed diets either the protein part or the free amino acid part was labeled. The postprandial oxidative losses of dietary leucine after 5 days being fed these diets (short-term adaptation) appeared to be significantly higher for the free amino acid diet compared to the protein diet. These differences between dietary free amino acids and dietary protein persisted in the mixed diets, as measured by the [$^{13}CO_2$] breath-test. It was concluded that amino acids derived either from a free amino acid or a protein diet, were handled independently even when ingested simultaneously during the same meal. Results obtained in rats were comparable to the results obtained in humans.
The differences in oxidation between a free amino acid and a protein diet had largely disappeared after long-term adaptation (after 26 to 30 days on the diet). An adaptive decrease in the oxidation of free amino acids was observed.
In the second study it was examined to which extent increasing levels of methionine supplements in a diet (50, 100 or 200% methionine supplement relative to casein) were retained in body protein. This was thus far not clear since a higher appearance rate in the free pool has been reported to have a negative influence on the efficiency of utilization of amino acids from the diet. Moreover, only specific patterns of amino acids are supposed to be deposited in body protein. Higher dietary methionine levels resulted in higher postprandial oxidative losses of methionine. The groups, which were fed the diets with the highest methionine levels, showed the lowest methionine retention as part of intake but the highest retention in absolute terms. After long term adaptation, however, to the free amino acid diets, methionine retention was increased in all groups. It was concluded that postprandial retention of dietary amino acids is, at least in part, driven by the amino acid composition of the diet.
In third study it was examined whether the postprandial fate of different dietary amino acid was regulated

by hormonal responses to the diet. It has been observed that the differences in oxidative losses between diets consisting of free amino acids or protein were not mediated by the combined action of insulin, glucagon, corticosterone and GH. Hence, postprandial catabolism of amino acids is probably regulated by other mechanisms.

As stated above, the amino acid appearance rate plays a crucial role in determining the postprandial utilization of amino acids. In the fourth study it was, therefore, investigated, whether the amino acid absorption rate can adapt to dietary free amino acids. Rats were kept on a free amino acid diet for 0 (non-adapted), 5, or 26 to 30 days (long-term adaptation). The methionine absorption of long-term adapted rats was lower than that of the non-adapted rats. It was concluded that the absorption of amino acid by the intestine plays a crucial role in minimizing the postprandial oxidative losses.

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Chapter 1

General Introduction

Preface

Proteins are, in general, the main dietary source of essential amino acids. Diets without an adequate amount of essential amino acids cause, in growing subjects, an impaired protein deposition and in non-growing subjects a wasting of proteins in the body. Minimal protein requirements are hard to establish since they are always modulated by short term alterations in physiological conditions. Long term protein requirements can be studied by the nitrogen balance technique. This technique studies, under steady-state conditions (e.g. nitrogen equilibrium or growth), the balance between the amounts of nitrogen ingested and excreted. This technique is not suitable to study short term changes in body protein metabolism within a day. The nitrogen balance technique indicates that a nitrogen equilibrium or growth can be achieved at different levels of protein intake (Quevedo *et al.*, 1994). If this is indeed the case, there must be metabolic differences at different levels of protein intake (Millward *et al.*, 1991; Waterlow, 1999b; Morens *et al.*, 2003). Those differences will become clear when focusing on the short term metabolic processes involved in maintaining the long term nitrogen balance. Important aspects of short term metabolic processes will be discussed in the following paragraphs.

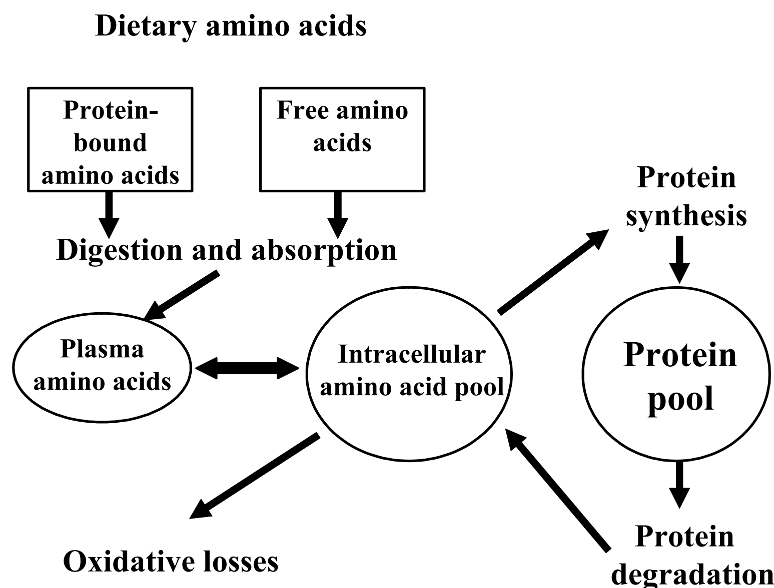

Fig 1. Short term metabolic processes involved in long term nitrogen balance.

Protein turnover

Body proteins are continuously degraded to form free amino acids (fig 1). These amino acids return to the intracellular pool of free amino acids (for review Millward, 1995; Millward *et al.*, 1996). This intracellular pool of free amino acids is continuous with the extracellular pool of free amino acids and with the free amino acid pool in the blood. Amino acids can exchange rapidly between these three pools (Wheatley *et al.*, 1986).

The amount of amino acids in these three free pools is under tight control. Due to the fact that high concentrations of amino acids, especially of indispensable amino acids, can be toxic the concentrations are kept low at a rather constant level (Erlandsen *et al.*, 2003, Funchal, 2005 #449). The main pathway to clear amino acids from the free amino acid pool is by the synthesis of body protein. The combination of protein degradation and protein synthesis is of physiological importance, being effective in the replacement of damaged functional proteins. Moreover, this combination allows a re-shuffling of amino acids between functional proteins in case of changing demands. In order to keep the size of the free amino acid pool constant, protein degradation and the protein synthesis should proceed at the same rate. During the turnover of body proteins, however, part of the free amino acids get lost by oxidation. These so-called un-avoidable or obligatory losses of amino acids have to be compensated by the intake of dietary protein (Millward, 1998).

Postprandial phase

The postprandial phase is the period directly after a meal when the diet is digested and absorbed by the body. Dietary proteins are digested to amino acids and small peptides. These amino acids and peptides are absorbed by the small intestine (review Ray *et al.*, 2002; Steffansen *et al.*, 2004)). The apical membrane of absorbing cells in the small intestine transports both free amino acids and peptides into the cell. The basolateral membranes of these cells, however, do not posses transporters for peptides. Once absorbed by the intestinal cells small peptides are further hydrolyzed to free amino acids. These free amino acids can be metabolized or catabolized by the intestinal cells or transferred to the circulation. The blood transports the amino acids to the tissues of the body where they join the extra- or intracellular free amino acid pool.

Dietary amino acids absorbed in free form cannot be stored as such. High concentrations of free amino acids are not only toxic, they also cause a tremendous change in the volume of the pool by osmosis. The only way to store dietary amino acids is a conversion to body proteins. This means that the pattern of dietary amino acids that is stored always reflects the pattern of one or more body proteins (Harper *et al.*, 1970). Most body proteins contain 20 amino acids but the

relative abundance may differ. In order to store dietary amino acids as a specific protein the entire amino acid pattern of that protein should be available.
To facilitate storage of dietary amino acids as protein, the nett protein synthesis capacity should be adequate. The nett protein synthesis could be increased by an increase in the rate of protein synthesis but is largely realized by a decrease in the rate of protein degradation (Gibson *et al.*, 1996; Millward *et al.*, 1996). Amino acids once stored as body protein, will further participate in the turnover of proteins during the postabsorptive phase.
The concentration of free amino acids in the body rises when the rate of absorption from the digestive tract exceeds the capacity of protein synthesis to store them. A rise in the concentration of free amino acids stimulates oxidative pathways to keep the levels within an acceptable range (Erlandsen *et al.*, 2003). A substantial postprandial oxidation of dietary amino acids, however, has a negative impact on their metabolic utilization. Therefore it is important to know how the postprandial storage of dietary amino acids can be optimized.
As long as the body is in N-equilibrium, the positive N-balance of the postprandial phase has to be counterbalanced by a negative N-balance in the postabsorptive phase. As mentioned before, N-equilibrium can be maintained at different levels of protein intake. A high protein intake causes a more positive N-balance during the postprandial phase, than a low protein intake, when stored accurately. This gives the body a broader scope to respond to changing demands. In conclusion the body can be in N-balance at different levels of protein intake, but a higher protein intake is more favorable for the adaptive capacity of the body.

Peptide and amino acid absorption

The prime products of intra-luminal protein digestion are oligopeptides. These oligopeptides are further hydrolyzed at the brush border membrane of the enterocytes in the small intestine. The resulting free amino acids, di- and tripeptides have to pass through the enterocytes in order to be reach the circulation (for review see Daniel, 2004). This is mediated by both active and passive transport mechanisms. a. The Ph gradient over the apical membrane is the driving force for the transport of small peptides into the enterocytes (Ganapathy & Leibach, 1985). Peptide transporters (e.g. PepT1) are located in the apical membrane but are absent in the basolateral membrane. Therefore, the peptides have to be further hydrolyzed within the enterocytes. Thereafter the resulting amino acids are either metabolized or catabolized by the enterocytes, or transported into the blood.
In the apical and the basolateral membrane there are different amino acid transporters located (Castagna *et al.*, 1997). The b^{0+} system is restricted to the apical membrane whereas the y^{+}L

transporter system is restricted to the basolateral membrane (Closs, 2002). This emphasis the different roles these transporters serve. On the one hand they support the nutritional needs of the enterocyte while on the other hand they are involved in the transport of the amino acids from the lumen into the circulation (Ray *et al.*, 2002). The intestinal cells use dietary amino acids to synthesize proteins. In this way up to 50% of the dietary amino acids is metabolized by the small intestine (Stoll *et al.*, 1998b; Wu, 1998; Lobley *et al.*, 2003).

Most mammalian amino acid transporters exhibit a broad substrate specificity (Soriano-Garcia *et al.*, 1999). They contain ten or more membrane spanning domains (for review see Palacin *et al.*, 1998; Steffansen *et al.*, 2004). These domains allow them to recognize, bind and transport amino acids in or out the cell. Most amino acids can, therefore be transported by different types of transporters. Those types include Na^+-dependent and Na^+-independent transporters (Munck & Munck, 1997) as well as Cl^--dependent transporters (Munck, 1997). Some transport systems have a narrow specificity whereas others have a broad specificity (Broer *et al.*, 2000). The $b^{0,+}$system is a Na^+-independent system that is able to transport cysteine and neutral as well as cationic amino acids (Munck *et al.*, 2000). The y^+L transporter is a Na^+-independent system for cationic amino acids and serves as a Na^+-dependent transporter for neutral amino acids.

This thesis studies the metabolic fate of methionine and leucine ingested during a meal. Methionine and leucine are neutral amino acids and are transported by the same transport systems (Soriano-Garcia *et al.*, 1998).

Amino acid appearance rate

During a day the dietary supply of amino acids is not synchronized with the demands of the body to let the various physiological processes take place. The daily supply of amino acids is normally restricted to a small number of meals. The ingested proteins are digested to amino acids, which are stored as body proteins during the postprandial phase. Amino acids not incorporated in body proteins will get lost due to postprandial oxidation. As a consequence at the end of the postprandial phase the body will be in a positive nitrogen balance.

Postprandial oxidation of dietary amino acids is closely related to the nett appearance rate of amino acids in the blood. This nett appearance rate is the nett result of the amino acid absorption from the small intestine on the one hand and protein synthesis capacity on the other hand. The capacity for protein synthesis can, however, only be increased by a higher daily protein intake. This indicates that whole body protein metabolism requires time for adaptation when the level of protein intake changes.

Intermezzo.

One may compare the situation in protein metabolism with a severe thunderstorm rain in the dessert. When the thunderstorm starts, rain falls in great quantities. The soil, which is completely dry, is not able to absorb the rain and the water will flow away. Although the soil is completely depleted of water, it is not able to retain the rain water. In other situations as in the region of Holten in the Netherlands the soil is always moist. In that case the water that comes down even during a mild rain shower is rapidly absorbed to be utilized by the plants.

In case of amino acid metabolism the situation is actually similar. The peculiar condition that an increase in dietary protein supply can decrease amino acid retention is called the 'protein paradox' (Moundras *et al.*, 1993; Morens *et al.*, 2001). An individual used to a low amino acid intake cannot handle large protein meals efficiently. In such a case the protein synthesis capacity is too low to handle the large amount of amino acids before they are degraded. This causes a less efficient utilization of dietary amino acids.

An optimal efficiency of amino acid utilization requires that the postprandial amino acid appearance rate does not exceed the nett protein synthesis capacity. The maximal amount of dietary amino acids that can become available for the protein turnover and protein gain, in the postabsorptive phase, is determined during the postprandial phase. It is, therefore, important to determine the appearance rate of the dietary amino acids and the factors that influence this rate. The molecular form of dietary amino acids is an important factor in the regulation of this process. Protein and free amino acids differ in absorption kinetics. This difference has a crucial influence on their metabolic fate (Boirie *et al.*, 1997; Metges *et al.*, 2000; Dangin *et al.*, 2001; Bos *et al.*, 2003; Bos *et al.*, 2005). In case of a higher amino acid appearance rate is the degradation of dietary amino acids stimulated by substrate induction. All processes involved in postprandial amino acid metabolism are well regulated. This regulation involves neuro-endocrine, humoral and behavioral factors (Liu & Barrett, 2002).

Hormonal regulation

In the postprandial phase the rate of protein degradation is reduced. As a consequence of this reduction the appearance rate of endogenous amino acids is lowered during the postprandial phase. Anabolic hormones, like growth hormone (GH) (Biolo *et al.*, 2000; Gröschl *et al.*, 2003), insulin like growth factors (IGFs) (Estívariz & Ziegler, 1997; Brameld *et al.*, 1999; Frost & Lang, 1999; Wheelhouse *et al.*, 1999; Vary *et al.*, 2000), testosterone and insulin, stimulate protein deposition (Ang *et al.*, 2000). This stimulation is mainly due to a reduction in protein degradation (reviewed by Rooyackers & Nair, 1997). The most well known hormone involved in this process

is insulin. In response to dietary amino acids, plasma insulin levels rise in a dose dependent manner (Floyd *et al.*, 1963; Calbet & MacLean, 2002). Especially the amino acids leucine, isoleucine and arginine are know for their effect on insulin release (Anthony *et al.*, 2000a; Anthony *et al.*, 2000b; Anthony *et al.*, 2001a; Anthony *et al.*, 2001b; Anthony *et al.*, 2001c; Anthony *et al.*, 2001d; Anthony *et al.*, 2002). A postprandial rise in insulin levels inhibits protein degradation in a dose dependent manner. An increase in the level of free amino acids may stimulate protein synthesis (Tessari *et al.*, 1987; Gibson *et al.*, 1996) in synergy with insulin (Shah *et al.*, 2000a). This makes insulin a proper tool to adjust nett protein synthesis to the appearance rate of dietary amino acids.

This thesis

The aim of this thesis was to increase the understanding of the processes that determine the utilization efficiency of dietary proteins. I have focused on the amino acid appearance rate, which is inversely related to the utilization efficiency of dietary amino acids. In this thesis I have tried to elucidate the mechanisms that the body uses to cope with dietary amino acid sources that cause a high appearance rate during absorption.

In the postprandial phase of a meal the oxidative loss of amino acids is compared in diets that have either free amino acids or protein as source of amino acids (chapter 2). These two molecular forms were chosen because of their practical relevance. Protein sources deficient in one or more amino acids according to the AIN recommendation (Reeves *et al.*, 1993) are often supplemented with free amino acids. In human nutrition in humans free amino acids are used for several applications (e.g. parenteral feeding, nutritional supplementation in sports).

There are several reasons to assume that diets with free amino acids and diets with protein as the single amino acid source, are physiologically not always equivalent. Apart from possible differences in gastric emptying, differences in gastrointestinal passage rate and the rate of intestinal absorption have been reported (Darcy, 1984; Rerat, 1985). Free amino acids can be absorbed without digestion in the proximal part of the small intestine. Proteins release their amino acids only after digestion. Protein derived amino acids will, therefore, be absorbed in a more distal part of the small intestine and consequently the whole process will take more time.

In chapter 2 I studied the metabolic consequences of feeding free amino acids. Both postprandial oxidative amino acid loss and the postprandial incorporation of dietary amino acids in body protein were assessed.

In chapter 3 I studied the effect of different methionine levels in 21% free amino acid diets, in relation to their relative level in casein. The diet with the lowest methionine level (50% of the

amount present in casein) gave rise to two problems in the body. The first problem was that the body needs a complete pattern of amino acids in order to be able to store dietary amino acids. In this diet methionine will most likely be the limiting amino acid. The second problem was caused by the low methionine level itself. Apart from being a protein precursor, methionine is also an important donor of methyl and sulfur (Farriol, 1991; Stipanuk, 2004). Initially, sufficient methionine will be available in the body pools, while at the longer term the body will have to adapt to the lower methionine levels.

In the chapter 4 and 5, I studied the regulatory mechanisms that could be involved in the adaptation of the body to free amino acid diets. Chapter 4 describes the hormonal responses, that mediate adaptation to diets causing a high amino acid appearance rate. In chapter 5 it is studied wether intestinal amino acid absorption is involved in the adaptation process.

Chapter 2

Postprandial oxidative losses of free and protein bound amino acids in the diet: Interactions and adaptation.

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Abstract

Postprandial oxidation of free or egg white protein derived amino acids supplied by a meal was studied using the [$^{13}CO_2$] breath test in rats, as well as in part in humans.
Thirty eight male rats were assigned to four dietary groups. The four diets only differed in their protein fraction. Diet I consisted of 21% egg white protein. For the breath test egg white protein, intrinsically labeled with [1-^{13}C]-leucine, was used as a substrate. Diet II contained the same amino acids as diet I, though not in protein form but in free amino acid form. Free [1-^{13}C]-leucine was used to label this diet. In addition 1:1 mixtures of both diets were used in two groups, in which for breath testing either the free amino acid or the protein fraction was labeled with [1-^{13}C]-leucine. The animals were tested following short-term (on day 5) and long-term adaptation (on day 20) after the diet was given for the first time. The human subjects were not allowed to adapt to the diets.
The postprandial oxidative losses at day 5 were significantly higher for the free compared to the protein derived leucine. This difference was observed between the diets with a single amino acid source as well as between the mixed diets. This difference had, however, largely disappeared at day 20, as a consequence of an adaptive decrease in free amino acid oxidation. The relative higher oxidative losses as observed in the free amino acid part of the diets, at day 5, were confirmed in the human study.
It is concluded that free and protein derived amino acids in the diet are metabolized independently, There are no indications for an interaction between the oxidation of protein derived amino acids and free amino acids.

Introduction

A generally accepted dogma in many nutritional studies is the so-called 'Dietary protein paradox' (Moundras *et al.*, 1993; Morens *et al.*, 2000; Tome & Bos, 2000). In this paradox it is hypothesized that high protein meals have a negative effect on the efficiency of protein accretion within the body. This is thought to be due to the fact that although the body is able to adapt to different protein levels (Metges & Barth, 2000; Morens *et al.*, 2001), the adaptational mechanisms are limited (Garlick *et al.*, 1999; Jackson, 1999).

The same hypothesis holds true for the digestibility of proteins. Proteins that are digested faster are utilized less efficient than proteins that are digested more slowly. Moreover the protein digestion rate negatively affects the amino acid utilization efficiency (Boirie *et al.*, 1997; Dangin *et al.*, 2001; Dangin *et al.*, 2002)

In order to meet with the tabulated amino acid requirements, free crystalline amino acids are commonly supplemented to diets based on proteins with an overall deficiency in one or more amino acids. This approach presumes that the supplemented free amino acids are utilized in the same way and with the same efficiency as the amino acids derived from dietary proteins. It is generally accepted that free amino acids can replace protein derived amino acids to support the basic needs for maintenance and growth. This does, however, not imply that the replacement of proteins by free amino acids is without any physiological consequences. On the contrary, this replacement may induce the use of alternative metabolic pathways or may even lead to metabolic and physiological adaptation. The physiological consequences of an exchange of protein bound amino acids by free amino acids are therefore hard to predict (Metges *et al.*, 2000).

In 1974, Batterham started a still ongoing discussion as to whether the nutritional value of free amino acids and protein in the diet is equivalent (Batterham, 1974; Forsum & Hambraeus, 1978; Officer *et al.*, 1997; Boza *et al.*, 2001; Daenzer *et al.*, 2001).

Unlike amino acids incorporated in protein, free amino acids do not have to be released from the protein. Therefore, the absorption is not necessarily delayed by the digestion process. Free amino acids can, therefore, be immediately absorbed in the first (proximal) part of the small intestine. Amino acids derived from protein, will have to be digested first and thus will become available over for resorption in a more distal part of the small intestine. The appearance rate of free amino acids in the blood is higher for dietary free amino acids than for protein derived amino acids.

Dietary amino acids are not stored as free amino acids, since the size of the free amino acid pool remains constant, even during the postprandial phase when the dietary amino acids enter the free amino acid pool in relative large amounts. Therefore, dietary amino acids must be stored

immediately in the form of body protein. When the amino acid appearance rate exceeds the protein synthesis capacity, the surplus in amino acids is removed by oxidation. A direct relationship between appearance rate and postprandial oxidative losses has been shown by Bos *et al.*, (2003). The appearance rate of individual amino acids will be different and as a consequence the metabolic fate will also differ between individual amino acids (Schreurs *et al.*, 1997; Metges *et al.*, 2000; Bos *et al.*, 2003).
Since the bio-availability and utilization efficiency of different amino acid sources are difficult to predict, detailed metabolic tracer studies are required to determine the physiological utilization of dietary amino acids in case of specific feeding strategies.
In this study we have tried to gain insight in the physiological consequences of feeding diets with different amino acid sources. In two homologous experiments in rats and humans, the postprandial oxidative losses of free and egg white protein bound amino acids were studied using a $^{13}CO_2$ breath test. In the rat model the effect of short- (day 5) and long-term (day 20) adaptation to the diet was assessed. The human model was used for two reasons, firstly to see whether the rat results can be extrapolated to humans, and secondly to include a group in the study that was, in contrast to the rats, not adapted to the diet. Since rats first have to adapt to a novel feed before they start eating regularly, it was not possible to include a non-adaptive rat group in the experiment. The combination of two different models in one study may also help to extrapolate the results of the present study to other species.
The dietary free amino acids or egg white protein were labeled intrinsically with [1-^{13}C]-leucine. The oxidative fate of both dietary forms of amino acids could in this way be traced (Evenepoel *et al.*, 1997). The potential interactions between free dietary amino acids and dietary protein were also studied by feeding the rats a mixture of both forms of dietary amino acids. To avoid dual labeling, the mixed diet was studied using two groups. In both groups only one of the dietary amino acid forms (free amino acids or protein) was labeled.
As differences in oxidation rate might be largely explained by the appearance rate, it was expected that the oxidation rate of protein derived amino acids was influenced by the free amino acids present in the same meal.

Material and methods

The rat study

This study was approved by the Wageningen University Animal Ethics committee. The experiment was conducted with 38 male Wistar (WU) rats (Harlan, Horst, The Netherlands) of about 300 g. The rats were housed individually in macrolon cages (38 x 26 x 14 cm) at a room temperature of 22 °C under a light/dark regime of eight hours dark and 16 hours light. The dark period was from 9:00 till 17:00 h. The rats were fed twice a day for 30 min, between 9:00 - 9:30 h and 16:30 - 17:00 h. The rats became familiarized to this schedule by feeding the animals 10 g rat chow (Teklad Global 18% Protein rodent diet; Harlan) per meal during the 2 weeks preceding the experiment. During the experimental period the animals received 8.5 g of the experimental feed per meal. This restricted amount was always completely consumed and adequate to support a normal growth pattern. Water was available *'ad libitum'*.

The molecular form of the amino acid source in the diets (21% w/w dry matter) though different (see table 1 and 2), was always based on the amino acid pattern of egg white protein (Evenepoel *et al.*, 1997). Diet I (EW100) consisted of egg white bound amino acids. For the breath tests egg white protein was used intrinsically labeled with [1-^{13}C]-leucine (Evenepoel *et al.*, 1997). The labeled egg white protein was produced by feeding chickens a protein diet deficient in leucine. Leucine was supplemented as [1-^{13}C]-leucine to the chickens. This labeled leucine was incorporated in the eggs. Diet II (F100) contained the same pattern of amino acids as EW100 but the amino acids were present in free form. Free [1-^{13}C]-leucine was used as intrinsic label in this diet. In addition both diets were mixed 1:1. For breath test analysis of these mixed diets, either the free amino acid fraction (F50), or the egg white fraction (EW50), was labeled with [1-^{13}C]-leucine.

Table 1. Dietary groups of the rat study

experimental diet groups	**amino acid composition**		**[1-^{13}C]-leucine in test meal**	**# rats**
	free amino acids (egg-white profile)	*protein (egg-white)*		
EW100	0.0 %	21.0 %	Egg-white	10
F 100	21.0 %	0.0 %	Free	10
F50	10.5 %	10.5 %	Free	9
EW50	10.5 %	10.5 %	Egg-white	9

Table 2. Composition of the non-protein part of the rat feed and the amino acid composition of the free amino acid and the egg white diets in both the rat and the human experiments.

Feed composition					
Glucose	63.9	Alanine	12.18	Lysine	12.18
Cellulose	50	Arginine	11.34	Methionine	7.35
Soybean oil	50	Aspartic acid	22.89	Phenylalanine	12.18
$CaCO_3$	124	Cysteine	3.78	Proline	8.4
$NaH_2PO_4.2H_2O$	34	Glutamic acid	25.62	Serine	15.54
$MgCO_3$	14	Glycine	6.72	Threonine	10.08
KCl	11	Histidine	4.62	Tryptophane	3.78
KH_2PO_4	105	Isoleucine	11.76	Tyrosine	8.40
Vit/Min. mix	22	Leucine	17.22	Valine	16.38
Total 79.0 g		**Total amino acids 21.0 g**			

Breath test procedure

After short-term(ST-Ada, 5 days) and long-term adaptation (LT-Ada 20 days) to the experimental diets, [$^{13}CO_2$] breath tests were performed. At the day of the breath test the rats were placed in an air tight cage at 8:30 h. After half an hour (at 9:00 h) a blank air sample was taken from the case using a 50 ml syringe. Immediately thereafter rats were transferred to a cage with fresh air. In this cage [1-^{13}C]-leucine labeled feed was available for 30 minutes. Over a period of five hours air samples were taken every 30 minutes and after each sampling the rats were put in a cage with fresh air. The CO_2 of the air samples was analyzed for [^{13}C]-enrichment by a Finnigan Delta C Mass Spectrometer (Finnigan MAT, Bremen, Germany).

In order to be able to estimate the total amount expired [$^{13}CO_2$] accurately , the CO_2 production of the animals during the meal and the postprandial period was also measured a day prior to the experiment employing identical nutritional conditions as during the experiment. To measure the CO_2 production by the animals, they were placed in a ventilated cage (1ml/min). The ventilated air was discontinuously analyzed by a CO_2 analyzer (URAS 3G, Mannesmann, Hartman & Braun. Frankfurt am Main, Germany)

The human study

The human experiment was conducted with five healthy human subjects, two males and three females. The average weight of these subjects was 69 ± 9 kg, their length 180 ± 10 cm and age 22 ± 1 year. Except for the diet, the subjects were not allowed to eat or drink anything but coffee or thee (without sugar and milk) during the experiment.

The human diets had a macro nutritional composition similar to the rat diets. A meal (1796 kJ), contained: 860 kJ carbohydrate, (304 g apple sauce, Euroshopper, Netherlands), 668 kJ fat, (18.6g vegetable frying fat, Euroshopper, Netherlands) and 268 kJ amino acids in either egg white or free form, or a mixture of both forms (Table 2).

Each subject received each of the 4 diets twice, in a random order. Dietary habits of the subjects prior to the test were not checked, nor were they adapted or preconditioned to the experimental meals (N-Ada). After an overnight fast of 11 hours the subjects received their meal at 9:00 h. The meals were ingested within 15 minutes. Breath samples were taken by expiring breath into a vacutainer (10 ml) with a straw for 5 to 10 seconds. The breath was sampled over a period of six hours. Just before the meal was ingested, at 9:00 h, a blank breath sample was taken. During the first 2 hours, a breath sample was taken every 15 minutes, thereafter every 30 minutes. The meals were served to each subject in a different random order. CO_2 production of the human subjects was estimated based on their metabolic weight ($W^{3/4}$) using the Brody formula.

Breath test substrates

The breath test substrate used in this study was L-[1-^{13}C]-leucine (chemical purity of > 99%, isotopic enrichment > 99%) obtained from ARC (Amsterdam, The Netherlands). In the test meals in both the animal and the human experiment, part of the non-labeled leucine was replaced by labeled leucine. In case of free leucine labeling; 6.0 and 60.0 mg [1-^{13}C]-leucine was used per meal for the rats and humans, respectively. Labeled egg white was produced as described previously by Evenepoel et al. (1997). In case of the egg white protein diet (diet I), the total amount of labeled egg white contained 6.0 and 36.5 mg [1-^{13}C]-leucine for the rat and for the human experiment, espectively. The enrichment of the intrinsically labeled protein was measured by a Finnigan Delta C Ratio Mass Spectrometer.

Statistics

The data are shown as mean ± SE and analyzed by SPSS statistical program with a General linear model and post hoc Tukey test. Differences are considered to be significant when P < 0.05.

Results

Body weight development of rats

At day 0, the day the animals switched to the experimental diet the mean weight of the animals was 319 ± 2.1g (data not shown). The animals did not grow during the first 5 days of the experiment. During this period the feed intake by the animals appeared to be reduced. From day 6 onward, the animals ate their meal completely and started to gain weight again. At the end of the experiment at day 20 the average weight was 351 ± 2.6g. There were no significant differences between weight gain among the dietary groups.

[$^{13}CO_2$]-breath test in rats, short-term adapted (5 days) to the experimental diets

Diets with labeled egg-white protein (EW100 and EW50)

The recovery rate of label after ingestion of the egg-white meal, intrinsically labeled with [1-^{13}C]-leucine (EW100), gradually increased to a peak value of 4.4 % of dose/hour after 4 hours (fig 1A). After 5 hours at the end of the measurement period the recovery rate of the label was still about. 4.0 % of dose/hour.

The recovery of $^{13}CO_2$ from the 1:1 mixed diet in with the egg white protein was intrinsically labeled (EW50) did not differ significantly from the curve for the diet in which egg-white was the sole amino acid source (fig 1A).

Diets with labeled free amino acids (F 100 and F50)

The recovery rate of label after ingestion of a meal containing free amino acids labeled with free [1-^{13}C]-leucine (F 100) increased more rapidly than for the egg-white diet and reached a maximum value of 5.5 % of dose/hour already after 1 hour (fig 1A). Thereafter, the recovery rate remained constant for about 2 hours. Three hours after the diet had been given the recovery gradually decreased to a rate of 4.2 % of dose/hour. The recovery values of $^{13}CO_2$ from the 1:1 mixed diet in which the free amino acid part was labeled with free [1-^{13}C]-leucine were not significantly different from the meal with free amino acids only. The curves of both diets with intrinsically labeled protein, EW100 and EW50, were significantly different from the recovery curves of the groups labeled with free [1-^{13}C]-leucine, F100 and F50, between 0.5h and 3.5h after ingestion of the meal.

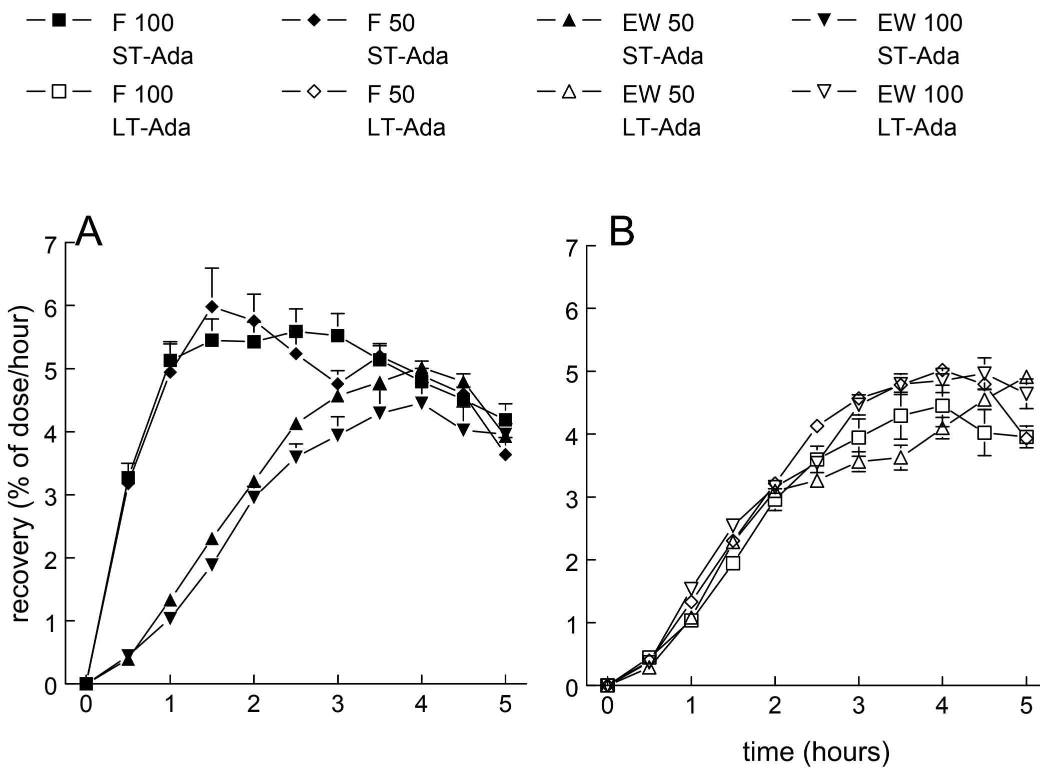

Figure 1. Postprandial oxidative losses, of diets with: 21% egg-white protein (EW100), 21% free amino acids (F100) or 1:1 mixtures of these diets (EW50 or F50) were estimated using a breath test in rats. The term "EW" or "F" refers to that part of the diet that was intrinsically labeled with [1-^{13}C]-leucine. The recovery is expressed as % of dose/hour ± SEM. Panel A: Short-term adapted (5 days). Panel B: Long-term-adapted (20 days).

[$^{13}CO_2$]-breath tests, Long-term adaptation (day 20) versus short-term adaptation to the experimental diets

Diet with egg-white protein

The recovery curves of both the EW50 and the EW100 group were similar to each other (fig 1B), both short- and long-term adaptation. In addition no significant differences were found between the short- and the long-term oxidative losses (fig 2A), neither for the EW50 group nor for EW100 group. The cumulative values were also similar (fig 3).

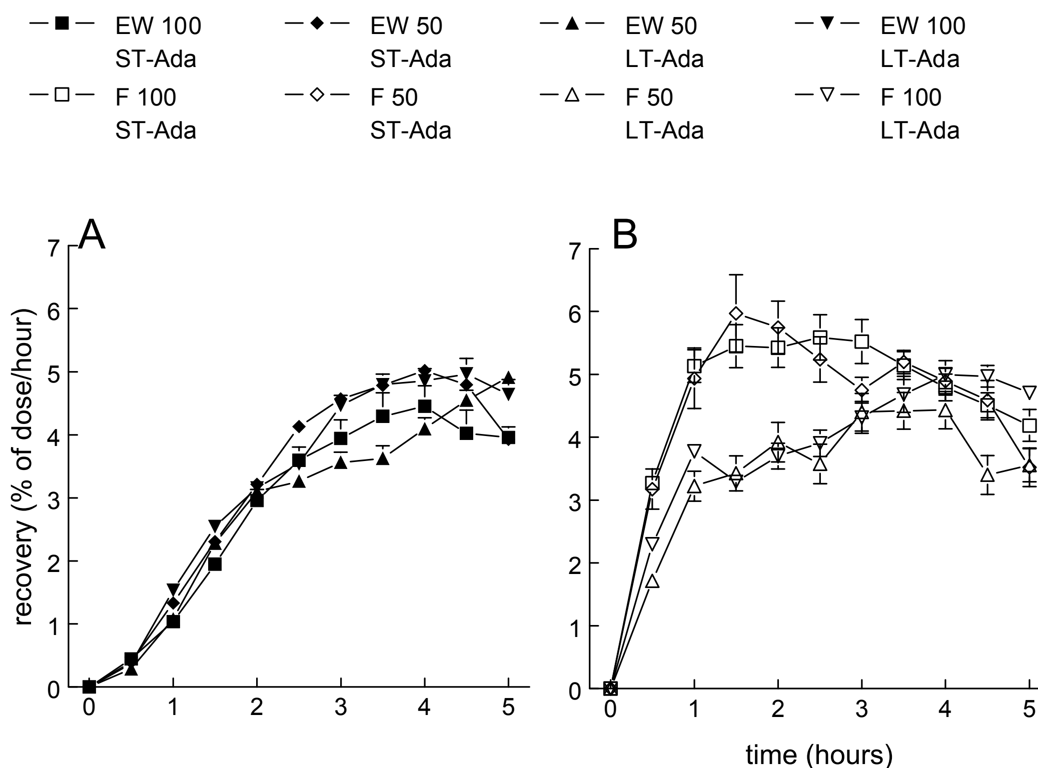

Figure 2. Postprandial oxidative losses, of diets with: 21% egg-white protein (EW100), 21% free amino acids (F100) or 1:1 mixtures of these diets (EW50 or F50) are estimated using a breath test in rats. The term "EW" or "F" refers to that part of the diet that was intrinsically labeled with [1-^{13}C]-leucine. The recovery is expressed as % of dose/hour ± SEM. Panel A; oxidative losses of protein derived amino acids of short-term (ST-Ada, 5 days on diet)and long-term (LT-Ada, 20 days on diet)adapted rats. Panel B; oxidative losses of dietary free amino acids of short-term (ST-Ada, 5 days)and long-term (LT-Ada, 20 days) adapted rats.

Diets with free amino acids

After long-term adaptation to the experimental diet, the cumulative recovery of label from free [1-^{13}C]-leucine was clearly different from the recovery pattern found after short-term adaptation (figure 1B, 2B and 3). The rapid increase in recovery during the first hour, as observed at day 5 was less pronounced after long-term adaptation. As a consequence, the rate of recovery at 1 hour was only 3.8 following long-term adaptation compared to 5.5 % of dose/hour following short-term adaptation. After this initial phase the rate of recovery increased slowly till a maximum value of 5.0 % dose/hour at t = 4 hours. The recovery rate remained more or less constant during the remaining of the experiment (fig 1B). The recovery of label from the F50 group at day 20 was similar to the F100 group, no significant differences were observed between these two groups.

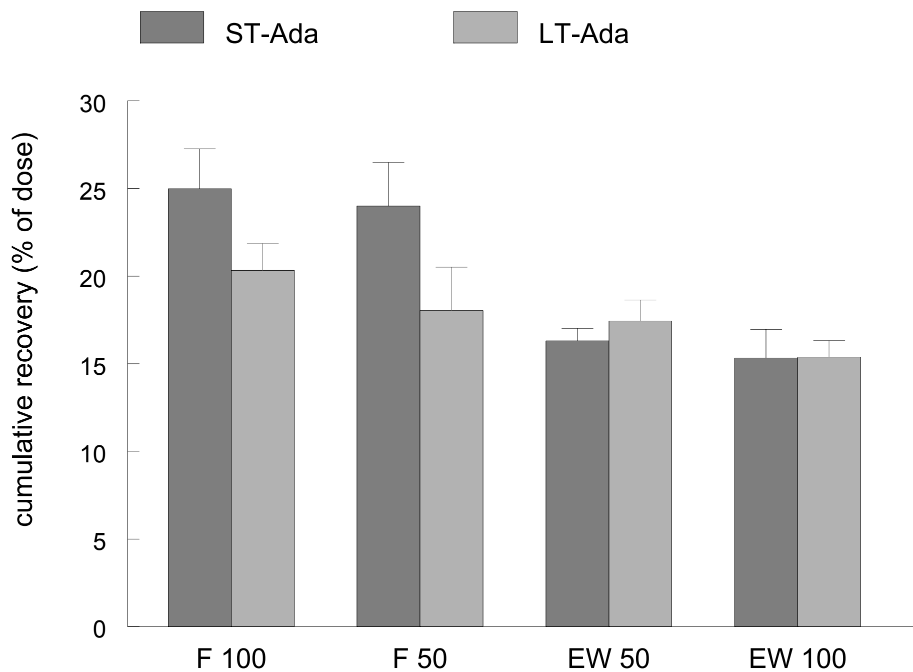

Figure 3. Postprandial oxidative losses, of diets with: 21% egg-white protein (EW100), 21% free amino acids (F100) or 1:1 mixtures of those diets are estimated with a breath test in rats. The term "EW" or "F" refers to that part of the diet that is intrinsically labeled with [1-^{13}C]-leucine. The data are expressed as cumulative recovery over the period of 5 hours (% of dose + SEM).

Postprandial amino acid utilization in humans (non-adapted)

egg-white protein diet

The recovery of label after ingestion of a meal containing only egg white protein (EW100) increased gradually after ingestion of the meal (fig 4); after 1 hour and 15 minutes a plateau level was reached of about 3% of dose/hour. Three hours after the meal had been given the recovery rate had began to decrease. At the end of the experiment the recovery rate was almost back to baseline levels.

No significant differences were observed between the recovery of label from the EW50 diet and from the EW100 diet.

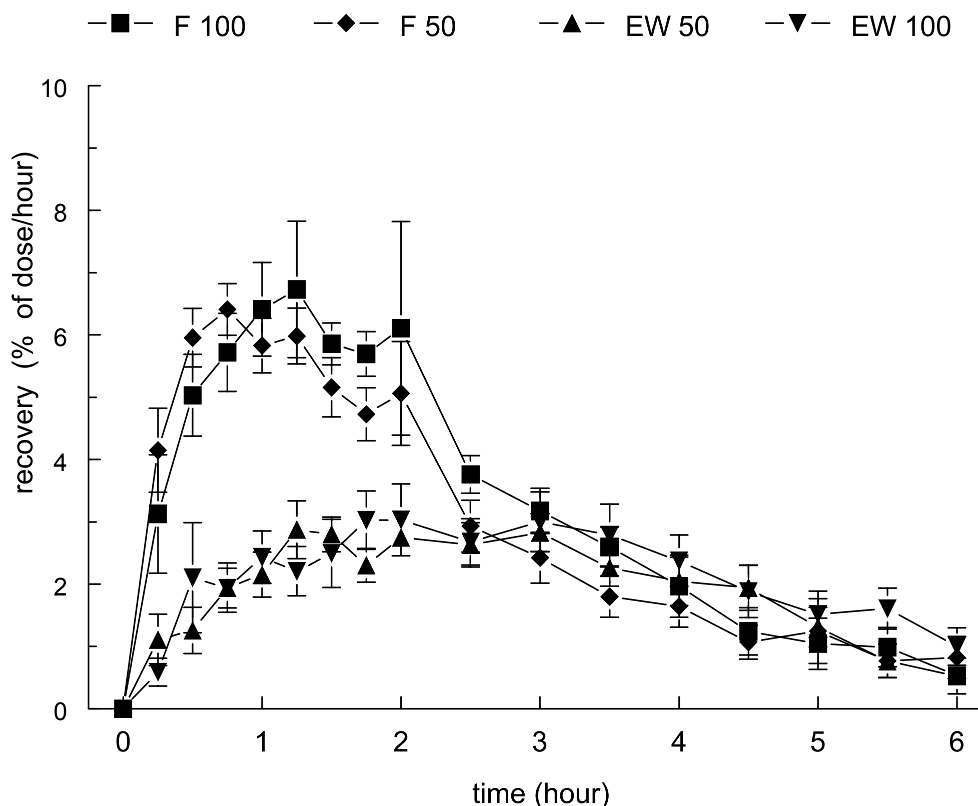

Figure 4. Human breath test. Recovery of $^{13}CO_2$ in the breath, expressed as % recovery of the dose/hour. Diet group labelled with free [1-^{13}C]-leucine is named F100 for the pure diet and F50 for the mixed diet. Diet group with [1-^{13}C]-leucine intrinsical labelled egg white is named EW100 for the pure diet and EW50 for the mixed diet.

Diet with free amino acids

The meal containing free amino acids (F100) caused a different recovery pattern (fig 4). The recovery increased rapidly to a maximal value of 6.7 % of dose/hour after 1 hour and 15 min. After 2 hours the recovery clearly began to decline and had returned back to baseline values by the end of the experiment.

The recovery of the mixed diet labeled with free [1-^{13}C]-leucine followed the curve of the 100% free amino acid diet labeled in the same way. No significant difference between these curves could be observed .

Discussion

The aim of the present study was to establish possible differences in postprandial oxidative losses between dietary free or egg white derived amino acids using a [$^{13}CO_2$]-breath test. Such differences could influence the relative nutritional values to the body of both forms of dietary amino acids.

Rat breath test results on day 5 after the start of the experiment indicated that the expiration rate of label from [1-^{13}C]-leucine was clearly related to its dietary form. The free amino acid diet with [1-^{13}C]-leucine showed a faster increase and a higher maximal value as compared to the leucine labeled egg white bound diet. At the end of the breath test, however, the expiration rate of label was similar for both dietary forms. The cumulative recovery of label from [1-^{13}C]-leucine was higher when dietary amino acids were ingested in free form (fig 3). This indicates that postprandial channeling of egg white derived amino acids to deposition in tissue, is more efficient due to a lower oxidative loss, confirming the results of (Metges *et al.* (2000) and Daenzer *et al.* (2001). Novel in the present study is th e diet in which the protein and free amino acid diets were mixed at a 1:1 basis. Followed by assessment of the oxidative losses of both the dietary free and the protein bound amino acid forms independently.
A surprising result of the underlying study was that the breath test measurements of labeled leucine free amino acid or protein diets, represented only that part of the meal that was supplied in the specific labeled dietary form. This indicated that the metabolism of free- and egg-white derived leucine, when present in the same meal, takes place independently. Comparing results for 100% and the mixed diets (consisting of 50% free amino acids and 50% protein; fig 1A) showed that postprandial metabolism of both dietary forms were, at least in this study, independent of their relative abundance.
This observation is puzzling because it implies that the body is able to discriminate between free and protein derived dietary amino acids. The small intestine would be the most suitable site for this discrimination to take place, since the small intestine is the only tissue that can clearly experience the differences between those dietary forms of amino acids. Amino acids from the free amino acid part of the mixed diet do not have to be released from the protein, but can readily be absorbed in the proximal part of the small intestine. Proteins need to be digested first before the amino acids are released and, therefore, it is likely that the resorption of these amino acids occurs at a more distal part of the small intestine and will take place at a later point in time.
It seems unlikely that post-absorptive organs, such as the liver, would have the ability to

discriminate between protein derived amino acids and free amino acids. Moreover, the hypothesis that the small intestine is able to discriminate between different dietary forms of amino acids, would be in line with other observations suggesting that the intestine plays an important role in the postprandial interaction of the body with different amino acid levels in the diet (Wu, 1998; Goudoever van *et al.*, 2000; Lobley *et al.*, 2003).

The breath test results of day 20 indicated that oxidative losses of dietary free amino acids decreased with time. The cumulative oxidative losses were lower following long-term adaptation compared to short-term adaptation (fig 3). This becomes particularly apparent in fig 2B which shows that the oxidative losses in the first hours after a meal are tempered. The maximal oxidation rate is lower and the peak value of oxidation is delayed for 2-3 hours.

The delay in maximal oxidation between short-term adaptation and long-term adaptation to the diet suggests that in the case of long-term adaptation the handling of free amino acids is slowed down, most probably in the digestive tract, thus reducing the appearance rate of the free dietary amino acids in the body pool.

Comparison of human and rat data

The human experiment confirmed the results of the rat study at day 5 following short-term adaptation, indicating that also in humans the free and egg white derived leucine was metabolized independently. In both species, the different amino acid components in the mixed diets showed no interaction. The recovery was only influenced by the nature of the label. For this reason it is hard to reconcile these results with the findings of Bos and colleagues (Bos *et al.*, 2003), who reported that the appearance rate was the main determent for the oxidation rate, unless the small intestine is able to cause differences in oxidative losses between the dietary free amino acid and dietary protein.

Beside the mentioned striking resemblance also some differences occurred between the responses of human subjects and rats to the experimental meals. In human subjects the response lasted for about two hours and thereafter the recovery values decreased to basal levels, whereas in rats the recovery rate did not return to basal levels. The meal size might influence the duration of the response. In this respect was the relative meal size of the rats (50% of the daily intake) probably larger than the size of the meal presented to the human subjects (<<50% of the daily intake).

It can also be argued that in the rat, a species with a higher metabolic rate than humans, the post-absorptive state and re-utilization of label becomes apparent earlier after the meal and is, therefore, more likely to interfere with post-absorptive processes.

In more detail, the F50, ST-Ada group showed a clear second peak in recovery of label, starting

3 hours after the meal, suggesting that probably re-utilization of dietary amino acids takes place. After long-term adaptation, all groups show a fast first increase in recovery during the first 2 hours after the diet. A transient peak was reached after 1 - 2 hours for both the free amino acid and protein labeled diets. A second increase was observed after 3 hours. We conclude that at the end of the measurement period the recovery curves for rats are already influenced by postabsorptive metabolism.

The differences between postprandial metabolism of free or egg white derived amino acids are observed in both growing animals (rat study) and in non-growing species (human study). This raised the question whether differences in postprandial metabolism should in growing animals always affect the growth rate. In the rat study, there was no significant difference in growth between the different dietary groups. Differences, as observed between the postprandial recovery rates were not reflected by differences in weight development. The growth rate is apparently determined by the combination of postprandial and postabsorptive metabolism. Possibly, the postprandial deposition of protein is only involved in the determination of the maximal growth rate. Whether the deposited protein is indeed actually used for growth, may depend on the circumstances. Higher postprandial oxidation rates are likely to be compensated for decreased oxidative losses in the postabsorptive phase. This could however impair maintenance functions.

In conclusion the present study has shown that free and protein derived dietary amino acids are metabolized in an independent way. Moreover there is no interaction between the oxidation of the free and the amino acid fraction derived from protein, when these two molecular forms of amino acids are ingested simultaneously with a meal. Initially a higher oxidative loss is observed for free amino acids during the postprandial phase. However, the body has a certain capacity to adapt to the presence of free amino acids in the diet as shown by a reduced oxidative loss after a period of approximately 3 weeks. The gastro-intestinal tract is the most likely site for this adaptation to occur.

Chapter 3

Metabolic Adaptation to Free Amino Acid Diets with Different Methionine Levels in Rats

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Summary

Postprandial amino acid metabolism has been studied in rats, using 3 different diets. The dietary amino acid pattern was based on casein (21% protein in diet). Methionine levels in the three diets were 50, 100 or 200%, relative to casein. L-[1-^{13}C]-methionine or L-[1-^{13}C]-leucine were used to trace decarboxylation by means of the appearance of [$^{13}CO_2$] in the breath as well as by the measurement of amino acid retention in tissue. Daily feed intake was divided in two distinct meals. The recovery of [$^{13}CO_2$] in the breath from L-[1-^{13}C]-methionine or L-[1-^{13}C]-leucine was assessed during 5 hours after the start of the morning meal. After these 5 hours also tissue amino acid retention was assessed. Growth was similar for all dietary groups, hence methionine was not a growth limiting factor. High dietary methionine levels caused higher [$^{13}CO_2$] recovery rates. Long-term adaptation (3wks) to the diets caused 1 hour delay in time of maximal recovery, for all dietary groups, compared to short-term adaptation (day 5). The cumulative recovery rate over 5 hours was similar for animals after short- and long-term adaptation. Dietary groups with 200% methionine, showed the highest absolute level of methionine retention in tissues, but lowest as part of intake. Methionine supplementation is thus reflected by higher postprandial methionine retention. Methionine retention in M. longissimus in adapted groups was 40% higher than in non-adapted groups. We concluded that postprandial retention of amino acids is, at least in part, driven by dietary amino acid composition.

Keywords
[^{13}CO2]-breath test, protein, amino acid metabolism, protein synthesis, regulation.

Introduction

In general the body will have a strong tendency to retain dietary methionine as well as other dietary amino acids in body protein. The synthesis of each protein chain starts with methionine. In addition, methionine also serves some other metabolic functions (Farriol, 1991); for example as sulphur-containing amino acid it serves a precursor for cysteine and glutathione. Up to 70% of the sulphur amino acid requirement in the body can be fulfilled by cysteine (Raguso *et al.*, 1999; Raguso *et al.*, 2000; Di Buono *et al.*, 2003). Methionine also plays a role in the methylation of several compounds (Raguso *et al.*, 1999; Raguso *et al.*, 2000; Di Buono *et al.*, 2003). The metabolic priority for cysteine in the body is about equal for glutathione synthesis and for growth (Baker, 1991). Whereas low methionine availability decreases growth (Rees, 2002) and reduces the health condition of a subject (Yen *et al.*, 2002), excess in methionine can also have negative consequences. It I has been reported that an elevated homocysteine level is a risk factor for vascular disease by several groups (Eikelboom *et al.*, 1999; Hankey & Eikelboom, 1999; Hofmann *et al.*, 2001). In a more recent study Troen and colleagues reported that not homcysteine but methionine is the causal factor for vascular disease (Troen *et al.*, 2003). The contradiction with the previous studies was explained by the observation that the height of homocysteine levels is highly correlated with methionine intake.
In well fed animals and humans many adaptational mechanisms to high dietary methionine levels exist (Finkelstein & Martin, 1986). However, in malnourished patients, the upper limit for a safe amino acid intake is lowered due to their poor condition (Jackson, 1999).
A general accepted dogma in many studies is the so-called ‘Dietary protein paradox’ which suggests that an amino acid abundance in the feed is reflected by a low utilization efficiency, due to a high appearance rate of amino acids in the blood and the tissues (Moundras *et al.*, 1993; Morens *et al.*, 2000; Morens *et al.*, 2001; Dangin *et al.*, 2002). Meals containing high amounts of protein amplify this effect, as they show this high appearance rate over a prolonged period of time (Morens *et al.*, 2003). In a similar way, meal size can influence the metabolic utilization of amino acids such as methionine and cysteine, of which it is known that in case of high dietary protein levels their oxidative loss is increased while their tissue incorporation is decreased (Tanaka *et al.*, 1990).
The aim of the present study was to investigate the decarboxylation and incorporation of amino acids in tissues after feeding growing male Wistar rats a diet consisting of different amounts of methionine. The results of this study should give an answer to the question whether the body stores dietary amino acids in accordance with physiological requirements (body driven), or does

the body try to store as much dietary amino acid as possible (feed driven). Animals were, therefore, fed one of three synthetic diets of fixed sizes, consisting of different amino acid patterns (50, 100 or 200% of methionine, compared to casein).

Material and methods

Two experiments were conducted, in which a casein-based free amino acid diet with three different levels of methionine was given. Postprandial oxidation as well as retention of dietary amino acids in several tissues was assessed using stable isotopes. In the first experiment L-[1-^{13}C]-methionine was used to trace both decarboxylation and tissue retention of methionine. L-[α-^{15}N]-lysine was also used to estimate tissue amino acid retention. In a second identical experiment L-[1-^{13}C]-leucine was used to estimate the effect of different methionine levels on total amino acid metabolism by measuring both leucine decarboxylation and its tissue retention. This study was approved by the Wageningen Animal Ethical Committee for Animal Welfare.

Animals

The two experiments with the different tracers were conducted using separate sets of animals. The metabolic fate of L-[α-^{15}N]-lysine and L-[1-^{13}C]-methionine was studied in 36 male Wistar rats. The metabolic fate of L-[1-^{13}C]-leucine was studied in 42 male Wistar rats. All rats were obtained from Harlan (Horst, the Netherlands). The rats with a mean body weight of 300 g were housed individually in macrolon cages (38cm x 26cm x 14cm), with sawdust bedding material. The daily feed intake was equally divided over two distinct meals of rat chow (Teklad Global 18% Protein rodent diet, Harlan). The meals were given at 9.00 and 16.30 hours. The animals were given 30 minutes to eat their meal. To allow adaptation to the new environment, light schedule and feeding regime persisted for at least two weeks previous to the experiment for all animals (fig 1). The dark period was from 9.00 till 17.00 hours.

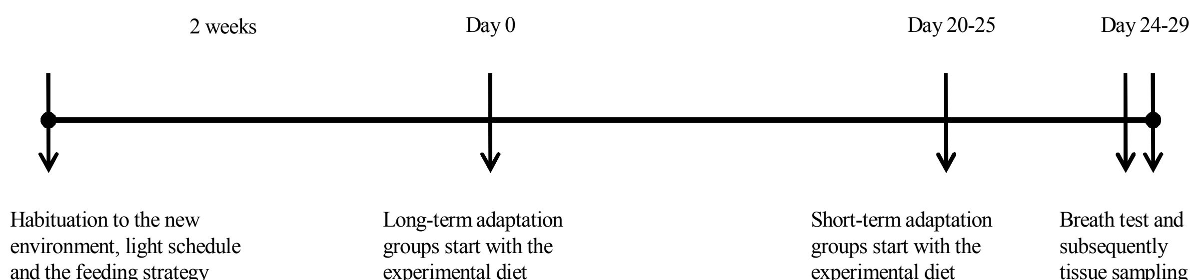

Figure 1. Schematic overview of the experiment.

After the 2 weeks of adaptation to the light schedule and the feeding regime, the animals in both experiments were assigned to 6 dietary groups stratified by weight. Three groups started this day with the experimental diet, to allow the animals to adapt to the novel feed for three weeks (long-term adaptation). Three other groups were continued to be fed the normal rat chow. To ensure that these rats would eat the whole experimental meal during the measurements, they were given the experimental diet for 4 days before the measurement (short-term adaptation). During the first days of adaptation to novel feed, animals ate less than normal. To avoid differences in feed intake between the long- and short-term adaptation groups, the meal size in all groups was adapted to the amount eaten by the least eating group. In three days the meal size increased to 8.5 g per meal, this amount was maintained throughout the experiment for all animals. The measurements were carried out at day 24-29 after the start of the experiment in a randomized order (fig 1). Water was available ad libitum. Daily feed intake, as well as body weight, were monitored, as parameter for the well being of the rats.

Table 1. Composition of the experimental diet and amino acid composition using the amino acid pattern of casein (100%), expressed as g/100 g feed.

Feed composition					
Glucose	63.9	Alanine	0.61	Lysine	1.81
Cellulose	5.0	Arginine	0.71	Methionine	0.59
Soybean oil	5.0	Aspartic acid	1.39	Phenylalanine	0.99
$CaCO_3$	1.24	Cysteine	0.53	Proline	1.79
$NaH_2PO_4.2H_2O$	0.34	Glutamic acid	4.64	Serine	1.18
$MgCO_3$	0.14	Glycine	0.36	Threonine	0.88
KCl	0.11	Histidine	0.57	Tryptophan	0.25
KH_2PO_4	1.05	Isoleucine	1.01	Tyrosine	1.09
Vit/Min. mix	2.2	Leucine	1.87	Valine	1.24
Total 79.0 g		**Total amino acids 21.0 g**			

Diets

All diets contained 21% free amino acids (w/w dry matter). The amino acid pattern was based on that of casein (see table 1). Within this pattern, only the methionine level was varied. Three different levels of methionine were used. The first diet had the casein pattern with a methionine level reduced to 50% of the level in casein (50% diet), the second diet had the same methionine level as normally present in casein (100% diet) and the third diet had a doubled methionine level (200% diet).

Tracer substrates

To avoid spillage of label from the experimental meal, this meal was prepared as porridge mixing 6 g of the experimental diet with 2 ml of water.

In all experimental meals (part of) the non-labeled amino acids were replaced by labeled amino acids, except for the experimental meal of one animal each dietary group. That animal was used to measure background values over the entire period of the experiment. In the first study, to assess methionine decarboxylation and retention in the tissues, L-[1-^{13}C]-methionine (isotopic enrichment > 99%, ARC; Amsterdam, The Netherlands) was used as tracer. In case of the 50% diet the amount of 17.6 mg methionine was completely replaced by L-[1-^{13}C]-methionine, in case of the 100% and the 200% diets 35.3 mg L-[1-^{13}C]-methionine was used. As far as relevant, 50 mg of lysine was replaced with L-[α-^{15}N]-lysine (chemical purity of 95%, VEB Berlin Chemie; Berlin, Germany). This second tracer was used to estimate the effect of different methionine levels on the retention of other dietary amino acids.

In the second study in all groups 20 mg of L-[1-^{13}C]-leucine (atom percentage 99%, Mass Trace; Woburn, USA) was used to trace both decarboxylation and tissue retention.

Breath test procedure

Postprandial decarboxylation of L-[1-^{13}C]-methionine and L-[1-^{13}C]-leucine was measured as recovery of [$^{13}CO_2$] in the breath during 5 hours after the start of the morning meal. At the day of the experiment, half hour before the start of the dark period, the rats were weighted and successively placed in an air tight cage. After half an hour a 50 ml air sample was taken from the cage. This sample served as a blank. Immediately after the start of the dark period two rats were transferred to a cage with the experimental meal and fresh air. Over a period of five hours air samples were taken every 30 minutes; after each sample the rats were transferred to a cage with fresh air. The CO_2 in the air samples was analyzed for [^{13}C]-enrichment by Isotope Ratio Mass Spectometric-analysis (IRMS, Finnigan Matt Delta C, Bremen, Germany) WIAS laboratory). For

the calculation of [^{13}C] recovery in breath total CO_2-production values are needed.
Values for the total CO_2-production are derived from the energy expenditure (kJ/day) estimated by using Brody's formula. The respiratory quotient (RQ) = 0.80 the EE (kJ/day) can be converted to CO_2 –production (l/day) using the conversion factor of 1.0 l CO_2 per 25.25 kJ. The Brody formula assumes that the basal metabolism (BMR) is proportional to the metabolic weight that is body weight $(W)^{0.75}$. For rats 400 x $(W)^{0.75}$ is used for calculating BMR (kJ/day). In the present experiment BMR values from the Brody formula are multiplied by correction factor (1.52 for the adaptation group and 1.18 for the non adaptation group) for activity and meal induced thermogenesis. These factors were derived from measurements of total CO_2-production prior to the breath tests day at day 23.
The CO_2-production measurement was performed in 2 or 3 rats from each dietary group (16 rats in total) from the experiment in which leucine was used as a tracer. The measurements were performed under conditions identical to the conditions of the [$^{13}CO_2$]-breath test, so meal induced thermogenesis was taken into account. Animals were placed in air tight macrolon cages (type II, 20 cm x 16 cm x 14 cm) with sawdust as bedding. Water was supplied ad libitum. The cages were ventilated with approximately 0.5 l/min. The air was dried with SPOCA (dried sponge with CaCl, thus absorbing water). CO_2 concentrations were assessed by a CO_2-analyzer (URAS 3G, Mannesmann, Hartman & Braun. Frankfurt am Main, Germany).

Tissue amino acid retention

Recovery of the tracers in several tissues was measured after terminating the breath test 5 hours after the the meal was supplied. Animals were anaesthetized with O_2/CO_2 mixture in a ratio of 2:1 after which the tissues were dissected From each animal up to 8 ml of blood was taken from the Vena cava inferior and the same volume of Ringer was returned. Subsequently, the femoral artery was cut and the animals were perfused with 40 ml Ringer through the Vena cava inferior. Liver, small intestine, kidneys, spleen, M. soleus and M. longissimus were dissected; in addition the contests of the small intestinal was collected. Sample material was immediately frozen on dry ice and stored at -20ºC till further analysis. For analysis the whole tissue samples were freeze dried and homogenized. The [^{13}C] enrichment in all samples was analyzed by IRMS (Finnigan Matt Delta C).

Statistical analysis

Breath-test recovery data are expressed as average percentage of the dose applied in L-[1-^{13}C]-methionine or L-[1-^{13}C]-leucine ± SEM, The data of amino acid retention in tissue and in

intestinal content are expressed as atom percentage excess ± SEM. Due to the fact that both the absolute amount and the relative amount of label in the experimental meals differed among the diets, the recovery data were corrected to make the dietary groups comparable. Statistical analysis was performed using SPSS 10.1. The model was an ANOVA model with diet as factor. Two different General Linear Models were used to test the differences; repeated measurements for the breath tests, and the multi variate model for the amino acid retention data. The Bonferoni test was used for Posthoc analysis. Differences were considered significant when $P < 0.05$.

Results

Weight gain

After the switch to the experimental diets the animals did not gain weight for the first five days (figure 2). All animals were feed restricted to the level of the least eating group. After those five days the animals of the adaptation groups were used to the new feed and ate their whole meal of 8.5g (data not shown). From that time on, all dietary groups resumed growth. There were no significant differences in weight gain between the experimental groups.

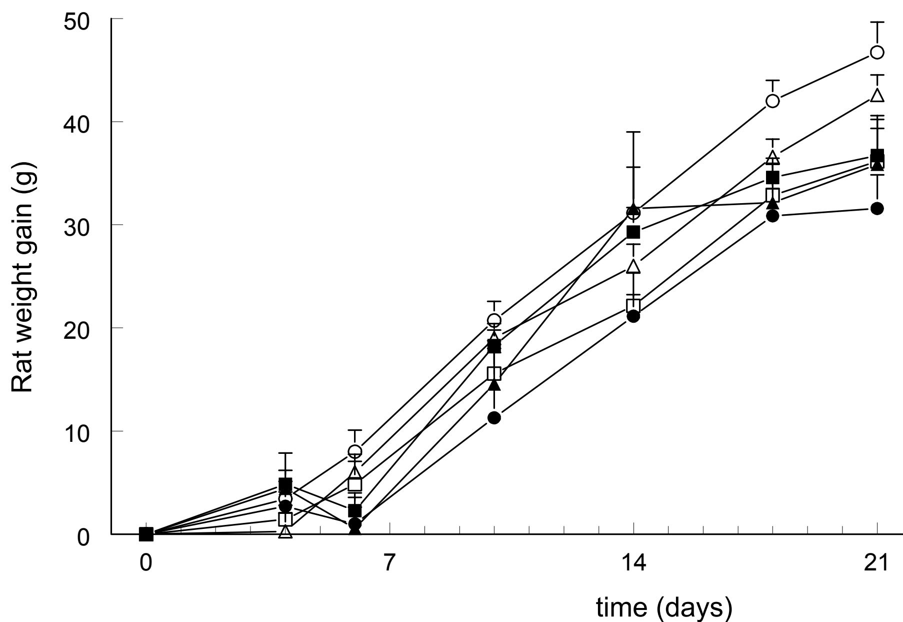

Figure 2. Rat weight gain (group mean + SEM) since the start of the experiment of long-(open marker) and short-term adapted animals (closed marker). Diets with 50% (squares), 100% methionine (circles) and 200% (triangles) methionine compared to casein.

Breath tests

L-[1-^{13}C]-methionine

The experimental amino acid meal induced, in all non-adapted diet groups, a rapid recovery of [$^{13}CO_2$] (figure 3A). Already 30 minutes after the start of the meal a significant part of the methionine label is recovered as [$^{13}CO_2$] in the breath. The recovery rate increased subsequently to reach a peak at time point t = 150 minutes after the meal. The [$^{13}CO_2$] recovery rate in the breath was higher for the diets with higher methionine content. The maximum values were 1.8 ± 0.1%, 2.7 ± 0.19% and 3.6 ± 0.38% of the dose per min for the 50%, 100% and the 200% group respectively. For the maximum values, the [$^{13}CO_2$] recovery was significantly different between all three dietary groups. After the peak at time point t = 150 the recovery curves of all three groups decrease gradually with the same speed. The curves did not reach the baselines before the end of the measurements at 300 minutes after initiation of the meal. The cumulative [$^{13}CO_2$] recovery values of the short-term adapted groups were 13.2 ± 0.6, 20.4 ± 1.1 and 27.8 ±1.3% of dose for the 50%, 100% and the 200% respectively (figure 4).

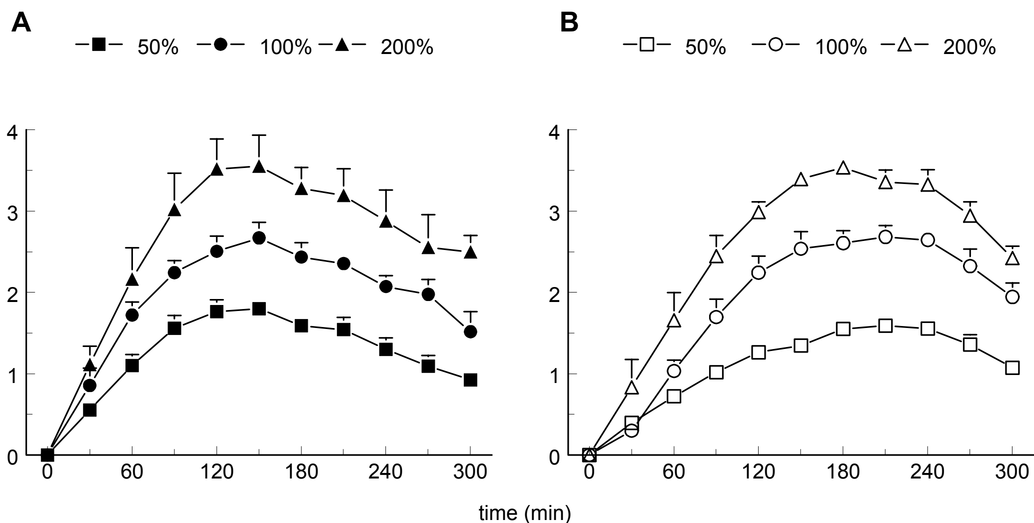

Figure 3. Postprandial recovery of [$^{13}CO_2$] in the breath from a meal labeled with L-[1-^{13}C]-methionine, expressed as % of dose/ 30 min at different times after the meal. Panel A; Short-term adapted groups. Panel B; Long-term adapted groups.

The metabolic responses to the free amino acid diets altered after an adaptation period of 3 weeks to the diet (figure 3B). As seen in the short-term adapted groups (figure 3A) the recovery of [$^{13}CO_2$] in the breath starts in the first sample after initiation of the meal. However, the slope, of the increase in recovery, is less steep, than in adapted animals. The peak values in adapted animals were reached with a delay of about 60 minutes compared to short-term adapted animals. This delay was significant ($p = 0.001$). But among dietary groups the peak values and the cumulative recoveries (figure 4) were similar to the corresponding values at day five. The peak values at time point t = 210 minutes were 1.6 ± 0.12, 2.7 ± 0.14 and 3.6 ± 0.15% of dose/30 min at time point 180 for the 50%, 100% and the 200% group respectively. The cumulative values of the LT-Ada groups were 11.9 ± 0.5, 20.0 ± 0.7 and 26.5 ± 0.9 % of dose for the 50%, 100% and the 200% respectively. The dietary effect for cumulative recovery was different ($p < 0.001$). In contrast, the effect of the adaptation period for cumulative recovery was not significant ($p = 0.454$).

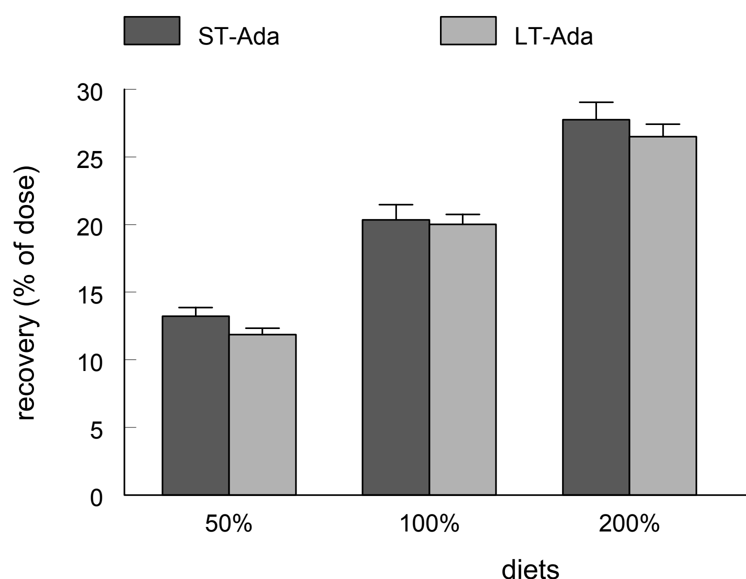

Figure 4. Cumulative methionine recovery of [$^{13}CO_2$] in the breath over a period of 6 hours after a meal, of rats adapted short-(ST-Ada) or long-term (LT-Ada) to free amino acid diets with 50%, 100% or 200%methionine compared with casein. Data expressed as percent of dose L-[1-^{13}C]-methionine. Measured by assessing the enrichment of carbon dioxide in the breath as a measure for methionine decarboxylation.

L-[1-^{13}C]-leucine

The recovery of [^{13}C] originating from L-[1-^{13}C]-leucine in all dietary groups, both the short- and the long-term adaptation groups, started immediately at the end of the meal (figure 5A and B), just as seen with the recovery of [^{13}C] from labeled methionine. For leucine, there was a sharp incline in recovery for the first 60 min followed by a less steep, but persisting incline until time point 270 minutes after initiation of the meal. Within the duration of the experiment no decline was seen in recovery of [^{13}C] originating from L-[1-^{13}C]-leucine. Although the recovery curve of the 200% short-term adapted group seemed lower, no significant differences with 50 % and 100 % were observed, (p = 0.316).

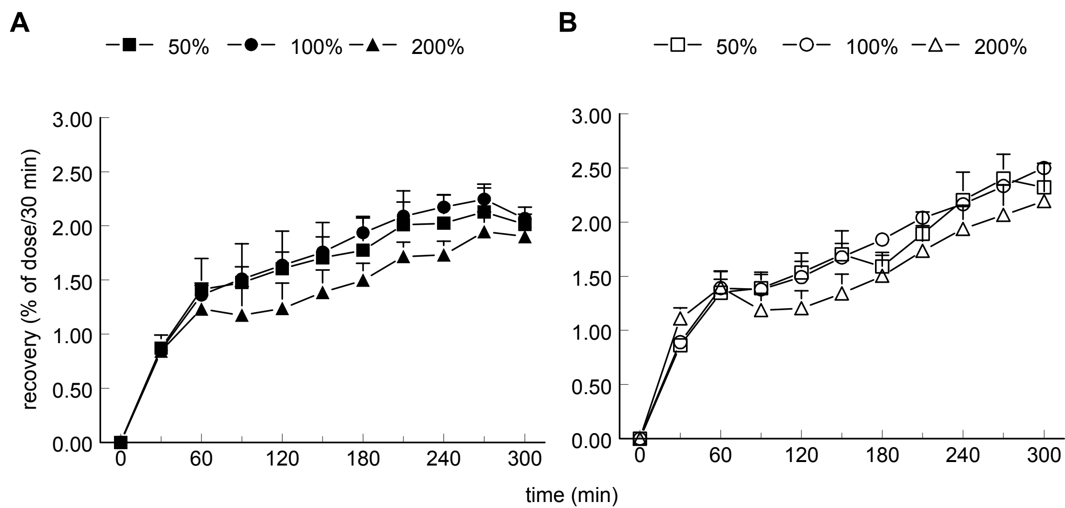

Figure 5. Recovery of [$^{13}CO_2$] in the breath, from a meal labeled with L-[1-^{13}C]-leucine, expressed as % of dose/ 30 min. Figure 5A; Short-term effects of a novel diet (day 5). Figure 5B; Long-term effects (3wks).

Tissue amino acid retention

L-*[1-^{13}C]-methionine*

In all organ and muscle tissues, the amino acid retention (expressed as tissue [^{13}C] enrichment) was significantly different between the three dietary groups (table 2). The L-[1-^{13}C]-methionine retention in the liver (figure 6) is shown as a typical example for all sampled organs. The 50% group showed the highest postprandial recovery of the label at 5hrs after initiation of the meal, for both the long- and the short-term group. The 200% group showed the lowest recovery of L-[1-^{13}C]-methionine. All dietary groups differed ($p < 0.001$). No significant differences between long- and short-term adapted groups were observed. So the adaptation period did not have an effect on the methionine retention in the liver.

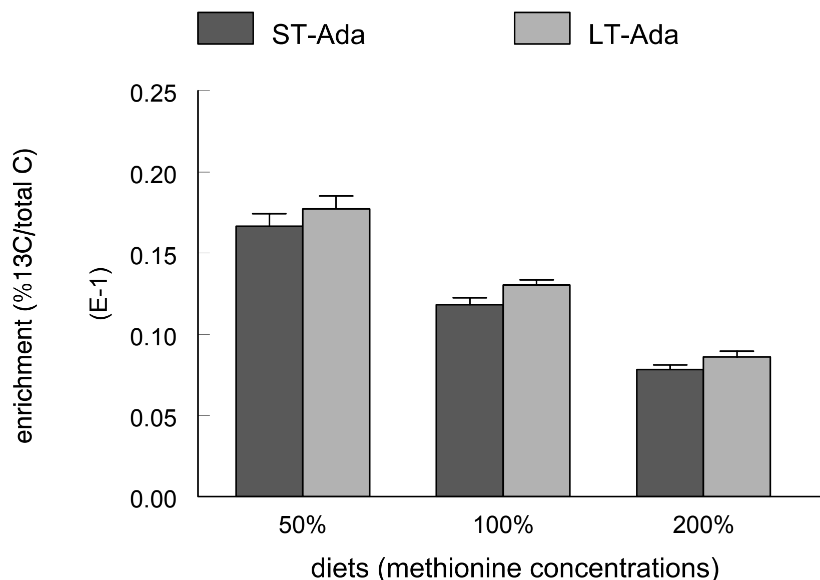

Figure 6. Enrichment of [^{13}C] in the liver 3 hours after a L-[1-^{13}C]-methionine labeled meal, expressed as percentage [^{13}C]/total carbon content x 10^{-1}. The methionine level of the 50% diet did only allow half the amount of L-[1-^{13}C]-methionine to be put in the meal in comparison to the 100% and the 200% diet. So enrichment is recalculated towards the same amount of label.

Table 2. Dietary L-[1-^{13}C]-methionine retention in different body tissues, expressed as atom %[^{13}C] /total Carbon content, in long-term adapted (LT-Ada) and short-term adapted groups (ST-Ada). These are the real enrichment values except for the 50% group. The methionine level of the 50% diet did only allow half the amount of L-[1-^{13}C]-methionine to be put in the meal in comparison to the 100% and the 200% diet. So enrichment is recalculated towards the same amount of label.

L-[1-^{13}C]-methionine										
diet	tissue	ST-Ada	SEM	LT-Ada	SEM	tissue	ST-Ada	SEM	LT-Ada	SEM
50%	liver	0.017	0.0008	0.018	0.0008	spleen	0.011	0.0005	0.012	0.0003
100%		0.012	0.0004	0.013	0.0003		0.0085	0.0003	0.0086	0.0002
200%		0.0078	0.0003	0.0086	0.0004		0.0058	0.0002	0.0065	0.0002
50%	kidney	0.015	0.001	0.018	0.001	M. Longissimus	0.0022	0.0002	0.0039	0.0001
100%		0.011	0.0006	0.013	0.0002		0.0019	0.0001	0.026	0.0001
200%		0.0074	0.0002	0.0085	0.0002		0.0014	0.00009	0.0023	0.0001
50%	small intestine	0.032	0.002	0.032	0.004	M. Soleus	0.0040	0.001	0.0048	0.0005
100%		0.021	0.002	0.023	0.0008		0.0026	0.0003	0.0033	0.0002
200%		0.014	0.0007	0.016	0.001		0.0020	0.0002	0.0022	0.0003

Muscle tissue shows diet dependent methionine retention (fig 7). Within the short-term adapted groups, the methionine retention of the 50% group was significantly higher, than that of the 200% group ($p = 0.011$). The recovery value of the 100% group in muscle was intermediate between the 50% and the 200% group (not significantly different from these two). There was, however, a very clear adaptation effect, in all dietary groups, for muscle tissue ($p < 0.001$).

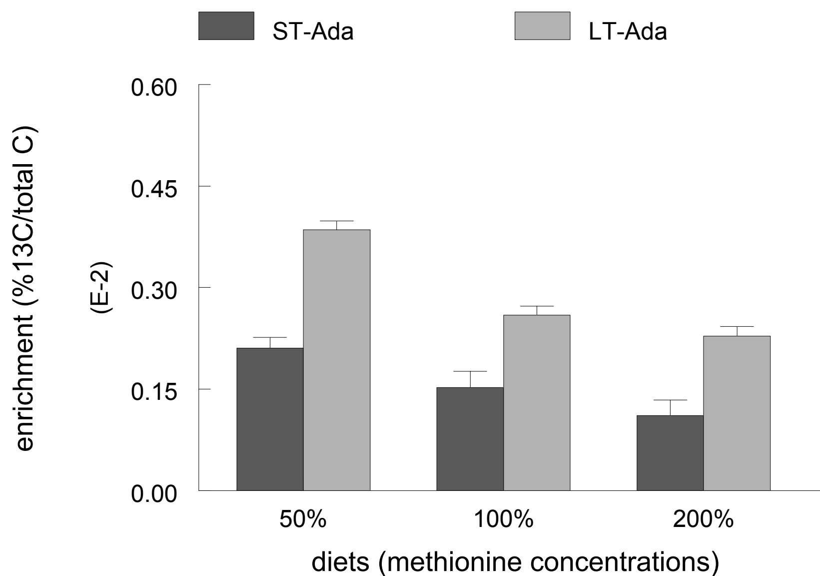

Figure 7. Enrichment of [^{13}C] in the M. Longissimus, 3 hours after a L-[1-^{13}C]-methionine labeled meal expressed as % [^{13}C]/total carbon content x 10^{-2}. Recalculated to compensate for the lesser amount of label in the 50% group.

L-[α-^{15}N]-lysine

Lysine was used as a second indicator for amino acid retention. The different dietary methionine levels had no effect on lysine retention (table 3). The liver is shown as a typical example for all organs (fig 8). The lysine retention in the liver was the same for all dietary groups. Organ tissue lysine retention was comparable with the muscle tissues and no significant differences were observed.

Table 3. Retention of dietary L-[α-^{15}N]-lysine in different organs and tissues, expressed as atom % [^{15}N]/total nitrogen content in animals long-term adapted (LT-Ada) and short-term adapted (ST-Ada) to the diet.

				L-[α-^{15}N]-lysine						
diet	tissue	ST-Ada	SEM	LT-Ada	SEM	tissue	ST-Ada	SEM	LT-Ada	SEM
50%	liver	0.083	0.003	0.083	0.002	spleen	0.036	0.002	0.039	0.0009
100%		0.088	0.003	0.083	0.002		0.035	0.003	0.037	0.001
200%		0.081	0.003	0.083	0.003		0.029	0.005	0.037	0.0006
50%	kidney	0.054	0.003	0.057	0.003	M. longissimus	0.012	0.0004	0.012	0.0003
100%		0.061	0.003	0.058	0.002		0.010	0.001	0.012	0.0006
200%		0.055	0.003	0.059	0.003		0.0092	0.002	0.012	0.0007
50%	small intestine	0.11	0.003	0.11	0.002	M. soleus	0.018	0.0003	0.018	0.0008
100%		0.11	0.008	0.12	0.003		0.015	0.002	0.018	0.001
200%		0.087	0.02	0.12	0.003		0.014	0.003	0.016	0.001

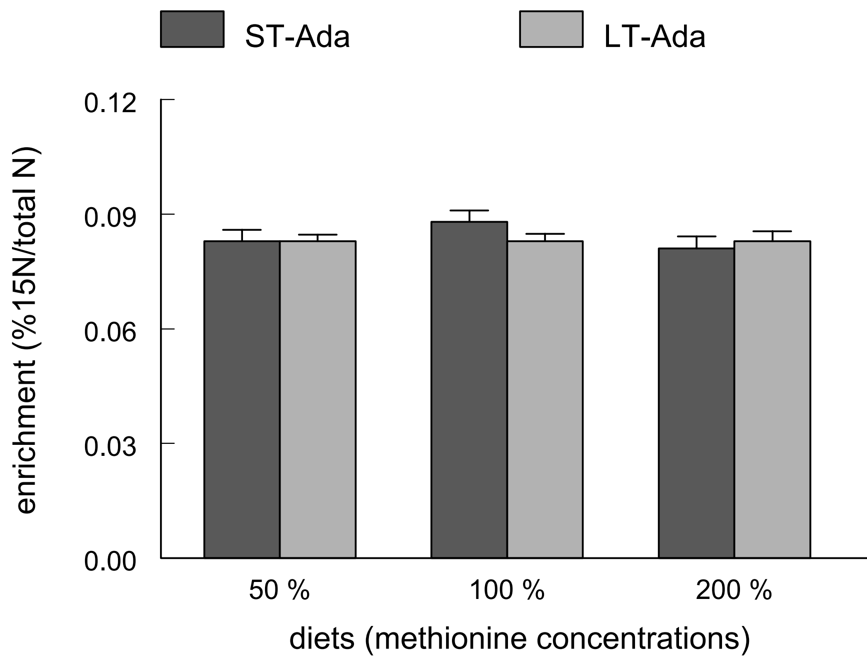

Figure 8. Enrichment of [^{15}N] in the liver 3 hours after a L-[α-^{15}N]-lysine labeled meal, expressed as percentage [^{15}N]/total nitrogen content.

L-[1-^{13}C]-leucine

Tissue L-[1-^{13}C]-leucine enrichment was different from the other tracers. The adaptation effect was significant in the kidney, spleen and M. longissimus (table 4). In these tissues the enrichments in the long-term adapted groups were decreased compared to the short-term adapted groups. There was no dietary effect, however, in any type of tissue.

Table 4. Retention of dietary L-[1-^{13}C]-leucine in organs and tissues, in animals long-term adapted (LT-Ada) and short-term adapted (ST-Ada) to the experimental diets. Data expressed as % [^{13}C]/total C. Organs and tissues marked with a * show a significant lower recovery in the adaptation group.

				L-[1-^{13}C]-leucine						
diet	tissue	ST-Ada	SEM	LT-Ada	SEM	tissue	ST-Ada	SEM	LT-Ada	SEM
50%	liver	0.0093	0.0003	0.0094	0.0004	*	0.0013	0.0001	0.0011	0.0001
100%		0.0088	0.0003	0.0093	0.0002	M. Longissimus	0.0013	0.0001	0.0012	0.0001
200%		0.0084	0.0003	0.0085	0.0007		0.0014	0.0001	0.0012	0.0001
50%	*	0.0072	0.0002	0.0070	0.0002	M. Soleus	0.0018	0.0001	0.0017	0.0003
100%	kidney	0.0074	0.0004	0.0071	0.0001		0.0019	0.0001	0.0016	0.0002
200%		0.0073	0.0005	0.0065	0.0003		0.0017	0.0002	0.0019	0.0002
50%	*	0.0060	0.0002	0.0059	0.0006					
100%	spleen	0.0062	0.0003	0.0055	0.0001					
200%		0.0058	0.0003	0.0051	0.0002					

Intestinal content

The recovery of [^{13}C] in the small intestine of the animal at the end of the experiment differed significantly between the different dietary groups. Post hoc analysis showed that the 50% groups were significant higher in recovery of [^{13}C] than the 200% groups. The 100% group did not differ significantly from the other experimental diets, but a clear trend is shown (fig 9). From these recovery values it can be calculated that 300 min after initiation of a meal, about 1% or less of the dietary amino acids can be found in the intestinal contents (not shown).

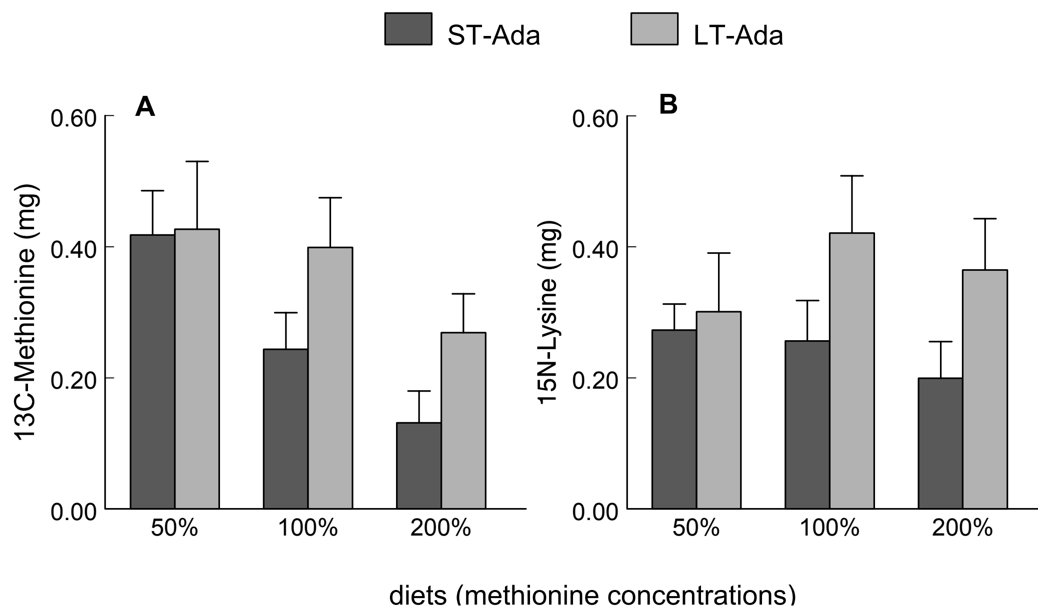

Figure 9. Amino acid content in the intestinal content. Panel A; The L-[1-^{13}C]-methionine content, expressed as mg L-[1-^{13}C]-methionine, recovered in the small intestine. The 50% group is recalculated to compensate for the lesser amount of label in this diet. Panel B; The L-[α-^{15}N]-lysine content expressed as mg L-[α-^{15}N]-lysine , recovered in the small intestine.

Discussion

Many dietary proteins are deficient in methionine according to the AIN recommendation (Reeves *et al.*, 1993). Methionine deficient diets are reported to affect health and growth performance. In many cases this effect on growth performance is at least partly caused by a reduced feed intake, associated with methionine deficient diets (Loughmiller *et al.*, 1998). In this study we have chosen for the amino acid pattern of casein which, according to the AIN norm, is low in methionine and has to be supplemented (Reeves *et al.*, 1993).

Growth performance

The dietary groups did not show any difference in growth performance. The 50% and 100% adaptation groups, which had too low methionine content in the diet for a prolonged time, grew as good as all other dietary groups. Although the 50% diet was assumed to be severe methionine deficient according to the AIN norm, it had no significant effect on growth performance. This suggests that methionine for growth was sufficient in the 50% diet. In the organs however methionine content of the tissue was affected by diet. This means organs which are considered to be associated with maintenance functions do not have a constant methionine requirement. Another option is that methionine is not the limiting factor. Due to the restricted feeding also the energy content could be a limiting factor for growth (Bikker *et al.*, 1994).

Breath tests

L-[1-^{13}C]-methionine

The recovery of [$^{13}CO_2$] in the breath showed clear differences between the diets. High dietary levels of methionine clearly increased the methionine decarboxylation rate. Since decarboxylation of methionine is irreversible, this implies a higher postprandial oxidative loss of methionine. The difference in cumulative recovery, however, was smaller than the difference in methionine level in the diet. So despite of a higher postprandial loss, still more methionine was retained and remains available for postabsorptive utilization with higher intake.

A higher oxidative loss does not mean, in the case of methionine, that the utilization efficiency has decreased. Decarboxylation of methionine does not exclude that it is utilized (via cysteine) for either protein synthesis or other functions like glutathione synthesis.

The recovery curves of the adaptation groups are less steep than the curves of the non-adaptation groups. The time at which the highest rate of recovery was reached, was on average, one hour later in the long-term adapted groups, compared to the short-term adapted groups. Since the

adaptation effects hold for all dietary groups, adaptation should be related to the free amino acid diets. It cannot be excluded that the differences in peak value are even higher for the first 3 days after receiving the test meals, than after short-term adaptation (day 5).
The rate of absorption or appearance rate is a powerful determinant for postprandial oxidative losses (Moundras *et al.*, 1993; Morens *et al.*, 2000; Dangin *et al.*, 2001; Morens *et al.*, 2001; Dangin *et al.*, 2002; Bos *et al.*, 2003; Morens *et al.*, 2003). A reduction in appearance rate should originate from processes in the gastrointestinal tract. The appearance of free amino acids can be delayed in time and/or the rate can be reduced. Both could explain the delayed [^{13}C] recovery in time as shown in fig 3 and fig 5. The values for the [^{13}C] recovery from L-[1-^{13}C]-methionine in the intestinal content shows especially in the short-term adapted groups an inverse relation between the dietary methionine level and the amount of tracer recovered in the intestine content. But all levels are low. The reduced recovery of [^{13}C] and [^{15}N] might be an indication that the amino acids are released at a lower rate by the stomach, or absorbed at a lower rate by the small intestine after eating high dietary levels. The absolute amounts recovered are at maximum less than 3% of the ingested dose. So absorption of amino acids can be considered complete. Despite the one hour time shift, in the breath test results, we assume that gastric emptying rate does not greatly influence metabolic availability of free amino acids.
A more logic explanation is that free amino acids will be absorbed more proximally in the small intestine, than amino acids released by digestion of dietary proteins. The small intestine is, at least in part, involved in the trapping and recycling of dietary amino acids. Under normal conditions, with amino acids bound in protein; these processes will take place more towards the distal part of the small intestine. Adaptation may cause a shift of absorption to the more distal part and this would be inline with a lower nett absorption or appearance rate.
This may be connected to a change in enzymes (protease) secretion into the lumen with the free amino acid diet after a few weeks. Those changes could be responsible for the delayed recovery as presented in figure 3.

L-[1-^{13}C]-leucine

The irreversible decarboxylation of leucine was assessed to study the influence of different methionine levels on the metabolic losses of other amino acids. The pattern of [$^{13}CO_2$] recovery from L-[1-^{13}C]-leucine in the breath is not affected by the methionine levels of the feed nor by the adaptation period.

Amino acid tissue incorporation

L-[1-^{13}C]-methionine

The tissue methionine retention results are at least in part complimentary to the results of the recovery in the breath. The dietary groups with the highest recovery of [$^{13}CO_2$] in the breath showed the lowest amino acid retention. This was the logical consequence of the fact that methionine decarboxylation is an irreversible process. The first priority of the body is to incorporate dietary amino acids in body proteins. The amounts that will or can be used for protein synthesis depends on nutritional and physiological conditions.
It is clear that the metabolic most active tissues showed the highest [^{13}C] enrichment. The intestine has the highest metabolic rate. This agrees the highest [^{13}C] enrichment found compared to other issues. M. longissimus showed the lowest [^{13}C] enrichment in the short-term adapted groups but the largest adaptation effect. So the nett increase in methionine retention of the whole body, as we derived from the breath test results, was paralleled by increased retention in M. Longissimus. This suggests that the breath test can be used to study changes in retention.

L-[α-^{15}N]-lysine

The [^{15}N] enrichment in all tissues was the same for all dietary groups both adapted and non-adapted. Apparently the extra storage of methionine in muscle was not associated with a higher retention of lysine. Therefore we concluded that the methionine levels have no effect on the protein synthesis rate. Because the methionine retention increased after the adaptation period, this means that the amino acid composition of deposition in protein must have changed.
Because growth was not affected, we also concluded, that free amino acids did not effect growth compared to protein.

L-[1-^{13}C]-leucine

The [^{13}C] enrichment in the experiment with [1-^{13}C] labeled leucine mimics mainly the results of lysine tissue incorporation in the first part of the experiment. There was no dietary effect of the varying methionine levels on leucine incorporation. This supported the conclusion, that methionine levels of free amino acid diets did not influence the protein synthesis rate or tha methionine at 50 % was not limiting.
The [^{13}C] enrichment values from leucine did show some significant differences. The kidney, spleen and M. longissimus enrichment of the adaptation groups was significantly decreased compared to the non-adaptation groups. This decrease was positive correlated with the methionine levels of the diets. This suggests that different methionine levels in a diet may cause

a redistribution of amino acids over tissues. The synthesis of proteins with a higher level of methionine was partly achieved (depending on tissue) by reduced leucine content.

Overall we can conclude that low methionine levels in the diet (down to 50 %) had no effect on weight performance. Supplementation of methionine increased the postprandial oxidative methionine losses. The whole body methionine retention however is still increased.

This study did not give a final answer about the mechanism of higher methionine retention. The tissue incorporation data showed that methionine supplementation as used in this study did stimulate methionine retention but did not stimulate the lysine and leucine retention. This suggests that the protein synthesis rate as a whole is hardly influenced. The elevated methionine retention might be due to the synthesis of proteins with high methionine content or due to an increased free methionine pool. As the free pool is normally strictly regulated and rather constant (Frontiera *et al.*, 1994), this is not the mechanism to be expected. The extra retention of methionine in the dietary groups with the higher methionine levels must than be due to the synthesis of proteins with higher methionine content (Bikker *et al.*, 1994).

Based on this experiment, it we conclude that the postprandial amino acid retention is at least partly determined by the feed (feed driven). The requirements will not change during the adaptation period, but due to adaptation the animals can store more dietary methionine during the postprandial phase.

Chapter 4

Dietary amino acids fed in free form and as protein components do not differently affect postprandial plasma insulin, glucagon, growth hormone and corticosterone responses in rats.

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Summary

This study examined, whether the postprandial fate of dietary amino acids from different amino acid sources is regulated by the responses of insulin, glucagon, corticosterone and growth hormone (GH). Male Wistar rats were cannulated in the vena jugularis and assigned to dietary groups. The diets contained 21% casein or the same amino acids in free form. In the free amino acid diets, methionine level was varied between the groups. The feed was supplied in 2 distinct meals. In previous experiments it was established that oxidative amino acid losses of the free amino acid diets and protein diets were different. After 3 weeks on those diets, it appeared that the differences in postprandial oxidative losses had been diminished. GH was measured every 12 minutes, from 144 min before the start of the experimental meal over the following 144 min. Insulin and corticosterone were measured 6 times from the start of the meal until 270 min after the meal. No differences were observed between the hormonal responses to both meals at day 5 and at day 26. In conclusion, it was found that the differences in the oxidative losses between protein and free amino acid meals are not mediated by the combined action of the insulin, glucagon, corticosterone and GH. Postprandial catabolism of amino acids is most probably regulated by substrate induction.

Introduction

Free amino acids are used for dietary purposes. In animal nutrition most dietary proteins are deficient in some essential amino acids. Therefore they are supplemented with these amino acids in free form. Also in clinical situations, parenteral feeding contains free amino acid mixtures instead of normal dietary proteins. Free amino acids can replace dietary protein to support the basic needs for maintenance and growth. However, this does not imply that the replacement of normal dietary proteins by free amino acids remains without physiological consequences. Metabolism has two options to cope with a dietary amino acid load; the amino acids can be incorporated into body protein, or oxidized directly. The latter mechanism becomes active when the protein synthesis capacity is not capable to utilize the whole amount of amino acids (Jean *et al.*, 2001). Potential consequences of a changeover between both dietary forms of amino acids are hard to predict (Forsum & Hambraeus, 1978; Officer *et al.*, 1997; Gaudichon *et al.*, 1999). The utilization efficiency of meals with free amino acids is lower or equal compared to meals with protein (Henry *et al.*, 1992; Officer *et al.*, 1997; Metges *et al.*, 2000; Daenzer *et al.*, 2001). The utilization efficiency of a free amino acid meal however, can improve in time (Nolles *et al.*, 2003). Thus, the body may modulate the utilization of dietary amino acids in time and metabolic hormones might be involved in this process.

Reports about insulin responses to amino acids date from the sixties (Floyd *et al.*, 1963). Glucose and amino acids may have a synergistic effect on insulin release (Rabinowitz *et al.*, 1966; Leclercq-Meyer *et al.*, 1985; van Loon *et al.*, 2000a; van Loon *et al.*, 2000b; Anthony *et al.*, 2002; Calbet & MacLean, 2002). Due to the synergistic effect of an amino acid load and the insulin response to a meal, in young animals, a meal intake stimulates muscle protein synthesis (Davis *et al.*, 1998; Davis & Reeds, 1998; Zhang *et al.*, 1999; Davis *et al.*, 2000; Davis *et al.*, 2001; Davis *et al.*, 2002). The postprandial effect of insulin is anabolic, either by a decrease of protein breakdown, or by an increase in protein synthesis. Insulin administration in the postabsorptive phase has a positive effect on protein gain (Ang *et al.*, 2000).

Amino acids induce an increase in insulin, growth hormone (GH) and glucagon, whereas corticosterone levels decrease (Shah *et al.*, 2000b; Tovar *et al.*, 2002; Gröschl *et al.*, 2003; Knerr *et al.*, 2003). In general GH increases whole body protein synthesis (Giustina & Veldhuis, 1998). The glucagon and insulin responses depend on the amino acid appearance rate in the blood (Calbet & MacLean, 2002), but high glucose levels inhibit the glucagon response to amino acids. Glucagon stimulates gluconeogenesis and consequently it stimulates withdrawal of especially the glucogenic amino acids from protein synthesis (Charlton *et al.*, 1996). Insulin and GH both have

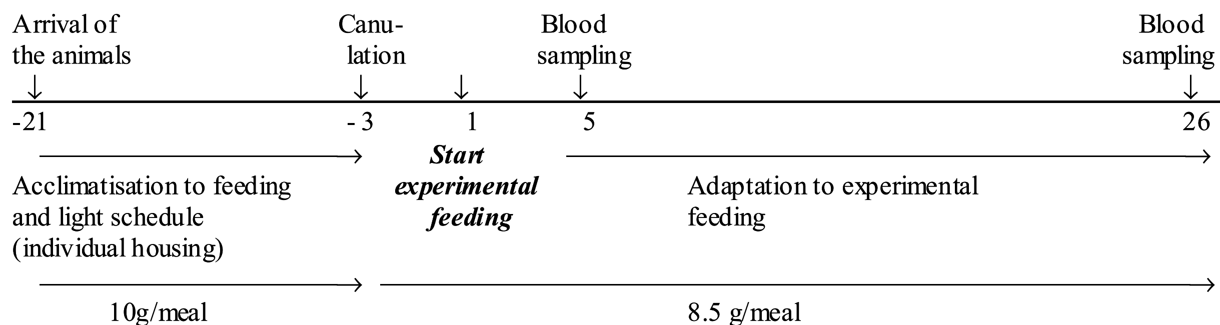
an anabolic effect on protein synthesis, whereas, glucagon and cortisol, or corticosterone in rats, have a catabolic effect (Tayek & Katz, 1997; Khani & Tayek, 2001; Gröschl *et al.*, 2003; Knerr *et al.*, 2003).

This experiment is designed to study the appearance of insulin, glucagon, growth hormone and corticosterone at various times before and after a meal. The present experiment is designed in a similar manner to a previous study in which the postprandial oxidative losses of dietary free amino acids were significantly higher than those from dietary protein (Nolles *et al.*, 2003). The goal of this study is to examine whether hormonal levels related to a meal are dependent on free or protein bound amino acids. In addition it was studied whether adaptation has taken place by measuring on day 5 and 26 after introducing the meals.

Materials and Methods

The hormonal responses to different free amino acids meals and protein meals were studied. The animals were adapted to the experimental diets for 26 days and the hormonal responses were measured on day 5 (short-term adaptation) and 26 (long-term adaptation) after introducing the meals (table 1). Day 5 was chosen because it was the first day all animals actually ate their experimental meal. On this day the animals were considered to be short-term adapted (ST-Ada). The two experiments were performed in a similar way unless otherwise stated.

Table 1. Experimental setup of experiment 1 and 2. The numbers indicate the days before or after start of experimental feeding.



In the first experiment, we measured hormonal responses to meals with 21% casein protein or the same amount of amino acids, supplied in free form. This study was performed with two similar batches of animals, with an interval of 6 months between the 2 batches. Both parts of this study were performed in the same way. After the second part, the samples for both parts were randomized and analyzed in the same assays. The results will be presented separately. In the case were the two batches differed significantly, the differences will be discussed in text.
In the second study we measured hormonal responses to three free amino acids diets, based on casein, differing in methionine level. In addition in both experiments a group received commercial rat chow as the animals received since weaning. The control group was needed to distinguish between adaptation to amino acid diets and a time effect between day 5 and day 26. Both studies were approved by the Wageningen University Animal Ethics committee.

Animals and housing

The first study was performed with 41 male wistar rats (table 2) and the second with 28 male Wistar rats (300 g). The animals were obtained from Harlan (Horst; the Netherlands). The rats, were group-housed in macrolon cages (55 x 33 x 18 cm), bedded with sawdust with a light schedule of 8 hours dark (red light, 4-5 Lux) and 16 hours light. The dark period was from 9.00 until 17.00 hours. The daily feed intake (20 g) was divided over two distinct meals (Teklad Global 18% Protein rodent diet; Harlan, The Netherlands). The meals were provided at 9.00 and 16.30 hours. The animals were given 30 minutes to eat each meal, which was sufficient time to finish the meal. The habituation to the new environment, light schedule and feeding regime persisted for at least two weeks for all animals (table 1). For both experiments, 5 male Wistar rats (± 400 g) were used as blood donors to replace blood after sampling.

Cannulation

After the habituation period the rats were cannulated in the jugular vein, according to the method of Steffens (Steffens, 1969). The day after the operation the cannulas were checked for proper functioning. To prevent the cannulas clotting, they were refilled with heparinized polyvinylpyrrolidon solution (1g PVP-25/ml saline; Merck, Darmstadt, Germany). After cannulation, the animals were housed individually in macrolon cages (38 cm x 26 cm x 14 cm), bedded with sawdust.

Table 2. Number of animals across batches 1 and 2 of experiment 1 within each dietary group. 4 diets are used; commercial rat chow diet (RC), 21% casein diet (CAS)and diet with the same amino acids but given as free amino acid (FAA).

Diet	batch 1	batch 2	Total
RC	7	6	13
CAS	7	7	14
FAA	8	6	14
total	22	19	41

Experimental diets

Immediately after cannulation, the animals were randomly assigned to the dietary groups, stratified by weight. In both experiments, a control group was fed commercial rat chow during

the whole experiment (RC). For experiment 1; the diet of the casein group (CAS) contained 21% casein (w/w dry matter), whereas the diet of the free amino acid group (FAA) contained the same free amino acids in free form (table 3).

Table 3. Complete composition of the experimental diets, expressed as g/100 g feed (w/w dry matter). Left column; all non-amino acid components. Middle and right column; The amino acid components for the diets of experiment 1 and the 100% methionine group of experiment 2. The 50% and the 200% group of experiment 2 consisted half, and double methionine levels as given in this table respectively.

Feed composition					
Glucose	639	Alanine	0.61	Lysine	1.81
Cellulose	50	Arginine	0.71	Methionine	0.59
Soybean oil	50	Aspartic acid	1.39	Phenylalanine	0.99
$CaCO_3$	124	Cysteine	0.1	Proline	1.79
$NaH_2PO_4.2H_2O$	34	Glutamic acid	4.64	Serine	1.18
$MgCO_3$	14	Glycine	0.36	Threonine	0.88
KCl	11	Histidine	0.57	Tryptophan	0.25
KH_2PO_4	105	Isoleucine	1.01	Tyrosine	1.09
Vit/Min. mix	22	Leucine	1.87	Valine	1.24
Total	**79**	**Total amino acids 21.0 g**			

For experiment 2, the animals were divided in one group fed control diet (RC) and three groups fed casein diet. The casein diets contained 21% free amino acids (w/w dry matter), with the same amino acid pattern as casein except for methionine (table 3). The diet of the second group (6 rats) contained 50% of the methionine level of casein (C50), the methionine level of the third group (7 rats) was equal to that in casein (C100) and the amino acid part of the diet of the fourth group (7 rats) contained double the methionine level of casein (C200).
The meal size was set to 8.5 g for all dietary groups in both experiments. The meals were eaten completely immediately after administration of the meal. This assured the same intake for all animals at the same time.

Two days after cannulation, the animals were assigned to the experimental diets. To avoid differences in intake between the dietary groups, during changeover to the experimental diets, the meal size of all groups was reduced to the level of the lowest eating group, which was 3.6 g

at the first day. Within three days the intended meal size of 8.5 g was being eaten by all dietary groups. This was a restriction of about 15% compared to ad lib feeding. For the rest of the experiment, the animals received 8.5 g per meal two times a day (i.e. 17 g a day), unless otherwise stated. The same procedure was repeated at day 21, to avoid differences in intake between day 5 and day 26.

Experimental procedure

Blood samples were taken on the 5th and on the 26th day after the start of the experimental diet. On these days, the rats received 6.0 grams of food to assure that the whole meal was eaten. The feed was presented from 9.00 until 9.30 (t = 0 is the start of the meal at 9.00 hours).

In experiment 1, blood samples (360 μl) were taken at -60, 0, 24, 60, 96, 144 and 270 min from feeding to measure insulin and corticosterone levels. Immediately after sampling the same blood volume was returned with blood from donor rats. For GH measurement blood samples of 70 μl) were taken at 12 min intervals from -144 before until 144 min after the start of the meal.

In experiment 2, an additional blood sample was taken during the first hour, this was undertaken as we sampled only at one time point of the insulin peak in the first experiment which in retrospect was insufficient. Blood samples (300 μl) were taken at -30, 0, 15, 30, 60, 90, 150 and 270 min from feeding to measure plasma concentrations of insulin, glucagon and glucose.

The samples were collected in heparinized tubes (5 IE/tube). Immediately after sampling, the samples were put on ice. As quickly as possible, the samples were centrifuged in a micro centrifuge for five minutes at 13000 rpm. The supernatant was pipetted and stored at -20ºC for further analysis.

Analyses

The GH, insulin, glucagon and corticosterone concentrations were determined by means of radio immune assays (RIA). Plasma insulin, glucagon and corticosterone concentrations were measured using rat RIA kits (Linco Research, Inc.)(Thorell & Lanner, 1973). GH levels were determined by a double-antibody RIA for rat-GH using materials supplied by NIDDK (rGH-I-6 as label and anti-rGH-S-5 as antiserum) and using Sac-cel (donkey anti-rabbit, Welcome Reagents, Beckenham, U.K.) as a second antibody. The levels of GH were expressed as area under the curve (auc). Glucose was determined with an enzymatic colorimetric assay (Roche Diagnostics, Inc. Basel, Switzerland).

Statistics

Statistical calculations were carried out using SPSS. For experiment 1; a general linear model, repeated measurements was carried out to test whether batch 1 and 2 differed significantly.

Insulin, glucagon corticosterone and glucose; a general linear model, repeated measures, was carried out to test whether the insulin levels of the dietary groups and the different measurement days differed. The diet and the day of measurement were the between-subject variables. For the adaptation effect, the animal was taken as covariate.

GH; GH-data were checked for normality By using normality plots. A ^{10}log transformation was performed on the GH data between the diets and between the measurement days to establish normality, A one-way analysis of variance (ANOVA) was carried out to test whether the mean of the auc levels of the dietary groups and the different measurement days differed.

Results

Experiment 1

Animal weight

The weight development of the animals in batches 1 and 2 were similar. Therefore the batches were combined (fig 1). No significant differences in weight gain were observed between the dietary groups.

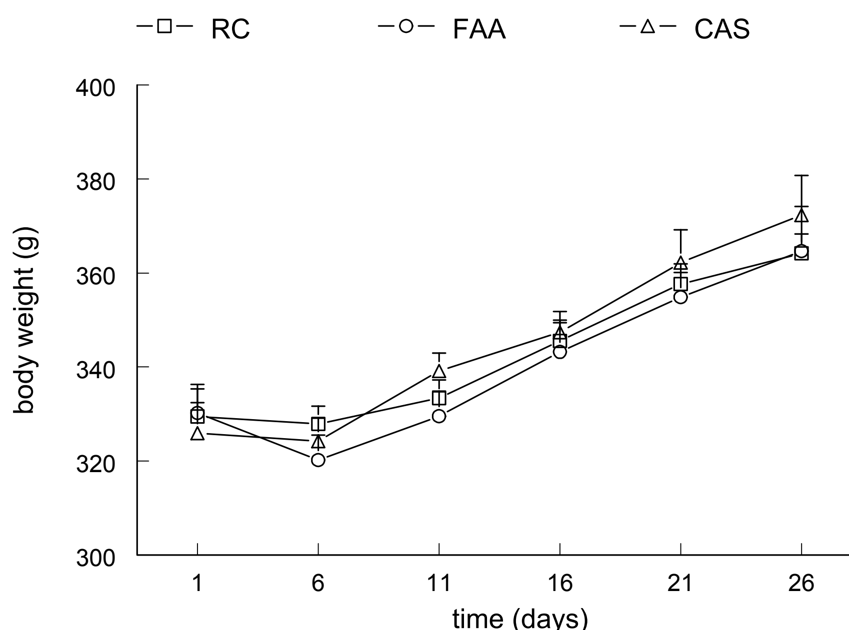


Figure 1. Rat body weight of experiment 1 (batch 1 and 2 combined) of the rat chow group (RC), the group fed diet with free amino acids with the pattern of casein as amino acid source (FAA), and the group fed diet with casein as amino acid source (CAS) expressed as group averages ± SEM (g).

Insulin

The insulin responses of the animals of the RC, casein and free amino acid groups were similar for all dietary groups (fig 2). This was the case on both day 5 and day 26, and there were also no differences between those days.

The peak levels for batch 1 were significantly higher than for batch 2 ($P = 0.013$). The average insulin peaks of batch 1 were 16.2 ± 3.3, 14.2 ± 2.7 and 11.5 ± 2.0 ng/ml for the RC, FAA and the CAS group respectively (Batches not shown separately). The peak levels for batch 2 were 11.4 ± 1.7, 9.2 ± 4.0 and 9.9 ± 1.8 ng/ml for the RC, FAA and the CAS group respectively.

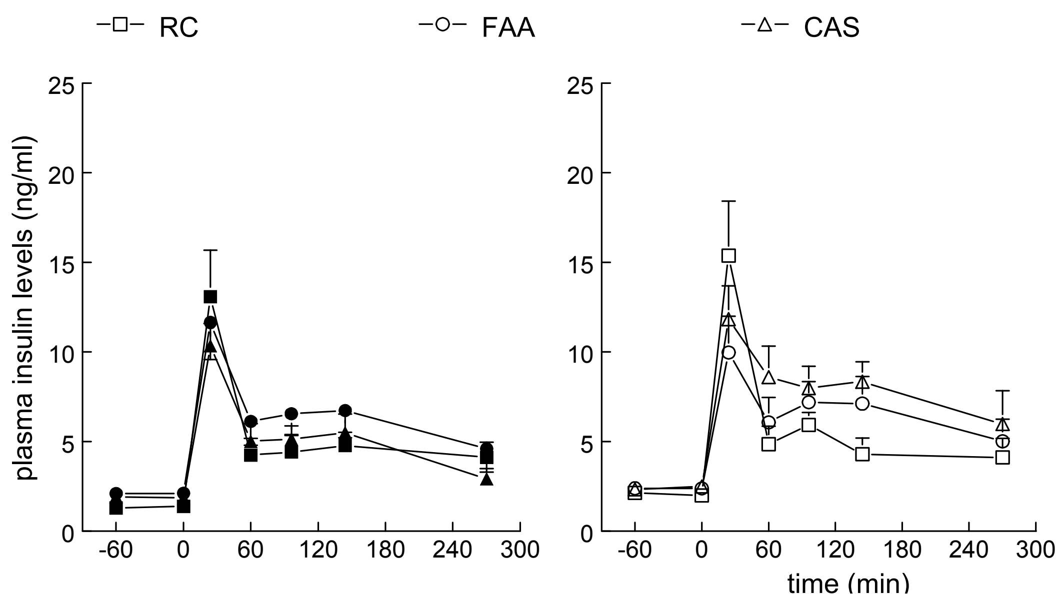


Figure 2. Plasma insulin levels of the rat chow group (RC), the group fed diet with free amino acids with the pattern of casein (FAA), and the group fed diet with casein as amino acid source (CAS), presented as group averages + SEM (ng/ml). At t = 0 meal was given and lights went off (start dark period). Panel A; insulin responses to the meal after short-term adaptation (day 5). Panel B; insulin responses to the meal after long-term adaptation (day 26).

Corticosterone

In figure 3, the plasma corticosterone levels are presented. The values of batch 1 and 2 were similar. Therefore, the results were combined. The RC group showed no differences between short- and long-term adaptation, so no interval effect was observed. There were no significant differences between the dietary groups. The corticosterone levels at long-term adaptation (fig 3B) were similar to the corticosterone levels at short-term adaptation (fig 3A). After long-term adaptation the corticosterone levels of the dietary groups were also not significantly different.

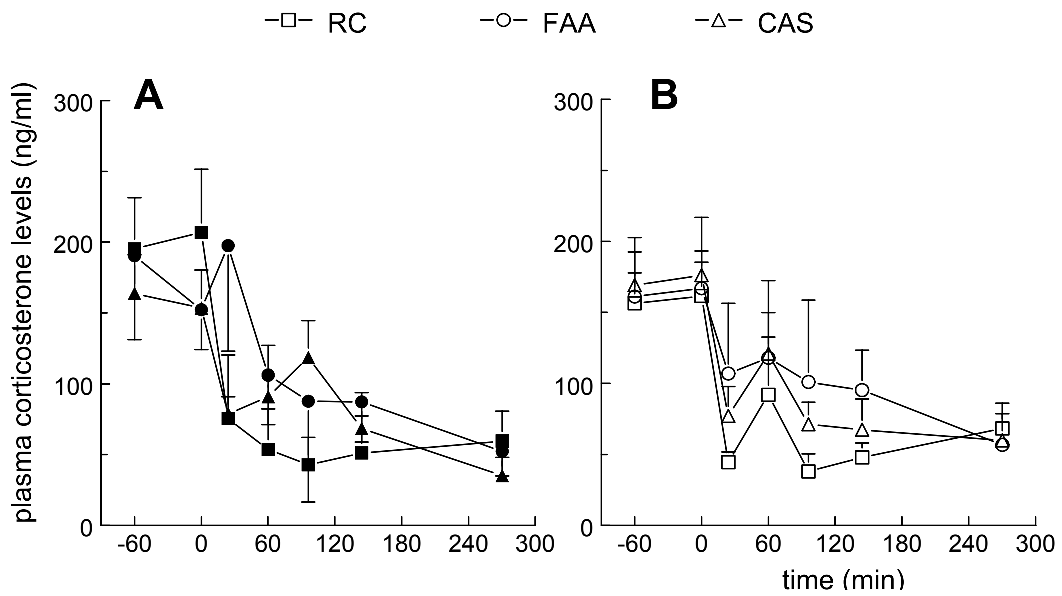


Figure 3. Plasma corticosterone levels ± SEM. Panel A; short-term adaptation (day 5). Panel B; long-term adaptation (day 26). Values of batch 1 and 2 combined. At t = 0 meal was given and lights went off (start dark period).

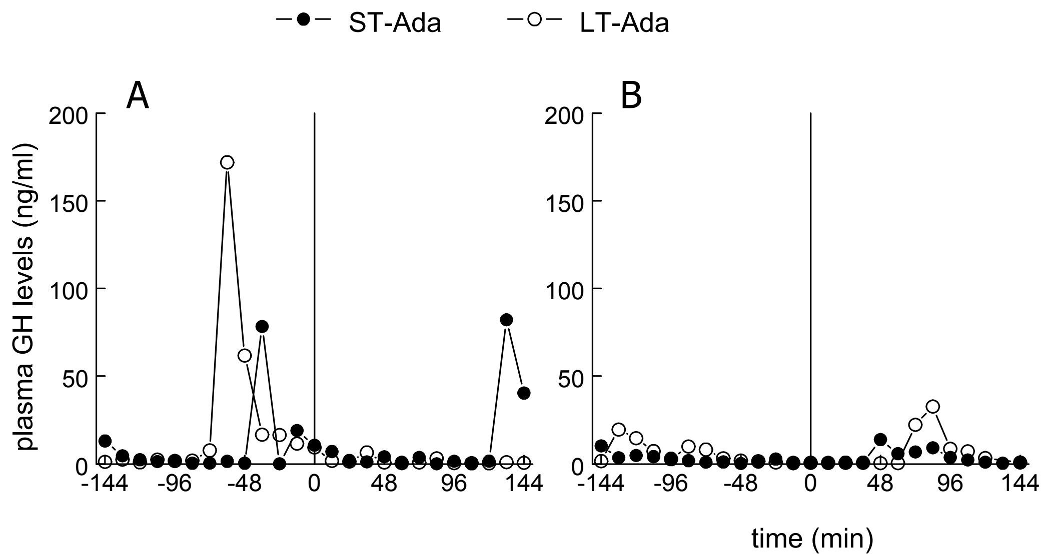


Figure 4. Typical examples of GH levels round the experimental meal after short- (ST-Ada) and long-term adaptation (LT-Ada), t = 0 is the start of the meal. Data are expressed as plasma GH levels ng/ml. Panel A; example of a rat with high GH peaks, Panel B; example of a rat with low GH peaks.

Growth Hormone

Most animals showed two GH peaks, one peak before the meal and one peak after the meal (fig 4). GH peaked at various time points and during the whole measuring period. The peak height varied between 20 ng/ml and up to 180 ng/ml (fig 4).

The animals fed the RC diet, showed no difference in level and pattern of GH, between day 5 and 26, so no interval effect was observed (fig 5). There were no significant differences in auc between short- and long-term adaptation, nor between the RC, casein and free amino acid groups.

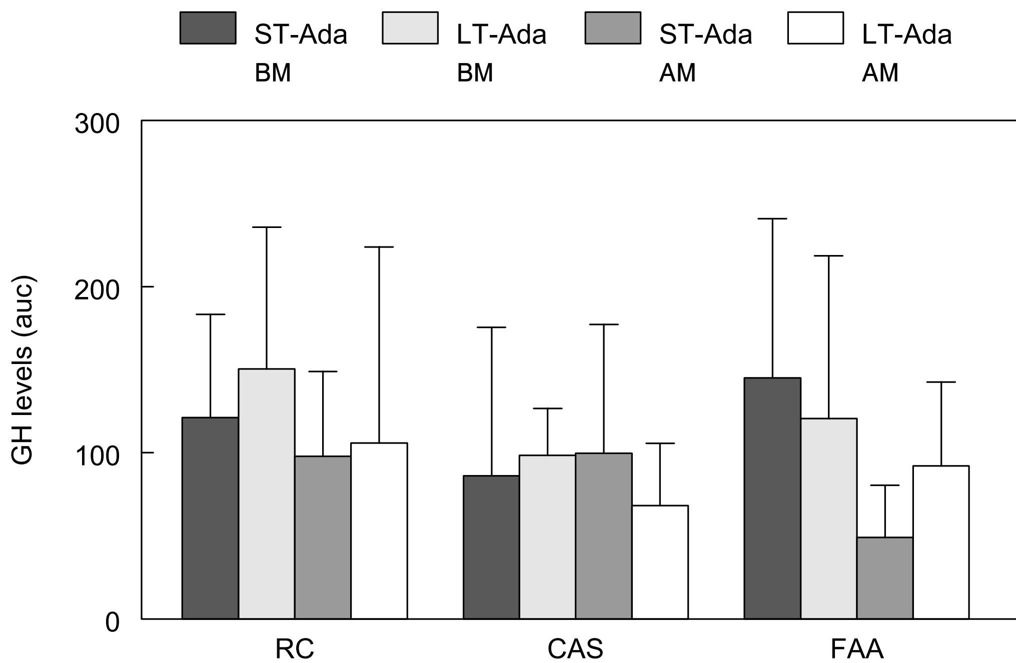
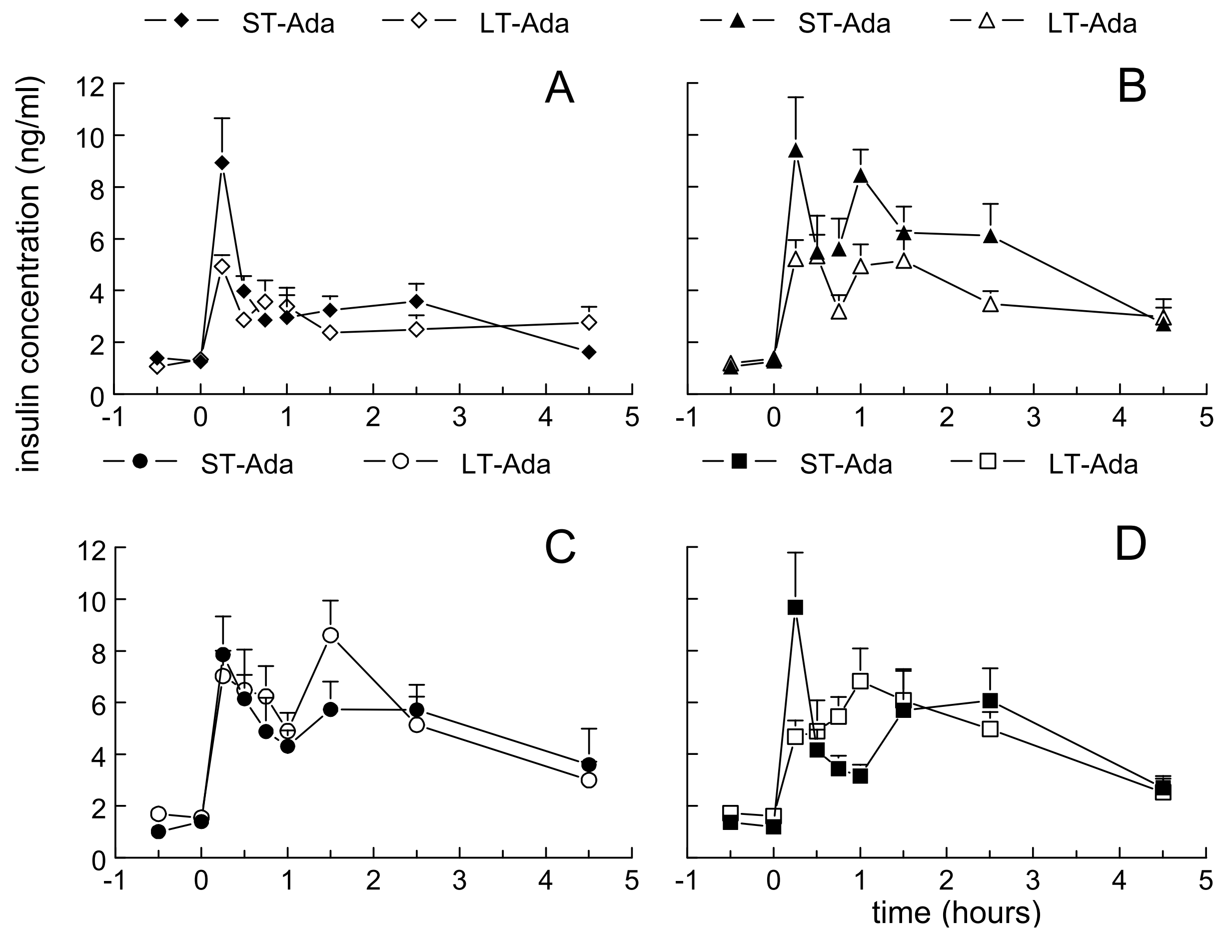


Figure 5. Plasma GH levels of the rat chow group (RC), the group fed diet with casein as amino acid source (CAS), and the group fed diet with free amino acids, with the pattern of casein, as amino acid source (FAA). Presented are the group averages + SEM of the area under the curve (auc) over the time period before the meal (BM), after the meal (AM) and over the whole experiment (tot) at day five (ST-Ada) and day 26 (LT-Ada).

Experiment 2

Animal weight

The animals weighted approximately 300 g when they were cannulated. After cannulation, these animals grew at about 3 g/day until the day the animals received the experimental diet. From that day, the animals lost weight for the next 5 days. After day 5 the animals continued to grow. After the long-term adaptation period (day 26), the animals weighted 340 ± 6 g. There were no significant differences between the dietary groups (data not shown).



Insulin

The RC group was significantly different from all free amino acid groups, but there was no significant difference between day 5 and day 26. No interval effect was observed (fig 7A). (fig 6 and 7). In contrast to the C100 and C200 groups, the C50 group seems to have a second insulin peak at 60 min, but no significant differences have been observed between the dietary groups (fig 7A).

The insulin responses at day 26 were similar to day 5 at least for the C50 and the C100 groups (fig 7B, 7C). The shape of the insulin responses of the C200 group was different from those of the other casein groups, values on day 5 and day 26 were not statistically different (fig 7D). At day 26, there were no significant differences between the insulin responses of the groups (fig 8B).

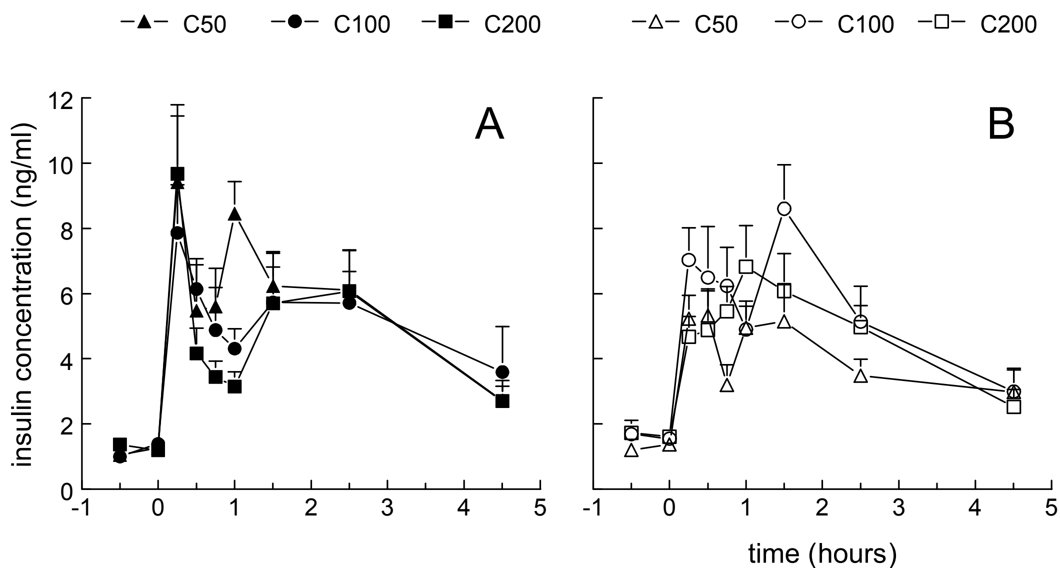


Figure 7. Plasma insulin levels of the group fed free amino acid diet with 50% methionine compared to the level of casein (C50), the group fed free amino acid diet with the casein amino acid pattern (C100), and the group fed free amino acid diet with 200% methionine compared to the level of casein (C200). Presented are the group averages + SEM (ng/ml). Panel A; short-term adaptation (day 5). Panel B; long-term adaptation (day 26).

Glucagon

The plasma glucagon levels of the RC group were similar for short- and long-term adapted rats (fig 8). No interval effect was observed. The glucagon levels of the RC group were also similar to the casein based free amino acid groups (data not shown).

At day 5, the plasma glucagon levels of all casein based free amino acid groups were similar (fig 8A), and the plasma glucagon responses at day 26 (fig 8B) were similar to those at day 5, no significant differences between the diet groups have been observed.

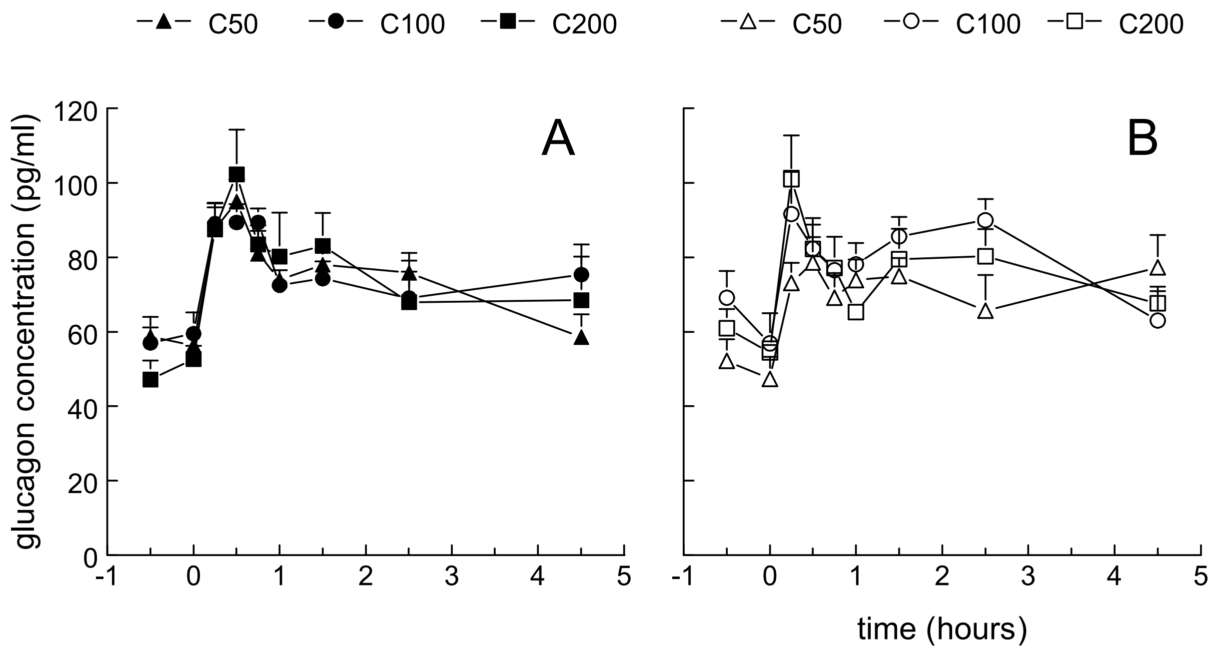


Figure 8. Plasma glucagon levels of the group fed free amino acid diet with 50% methionine compared to the level of casein (C50), the group fed free amino acid diet with the casein amino acid pattern (C100), and the group fed free amino acid diet with 200% methionine compared to the level of casein (C200). Presented are the group averages + SEM (pg/ml). Panel A; short-term adaptation (day 5). Panel B; long-term adaptation (day 26).

Glucose

The plasma glucose levels of the RC group were all similar at day 5 and 26 (data not shown). There were no significant differences between the RC group and the casein based free amino acid groups.

At day 5, the plasma glucose levels of animals with different dietary groups responded similarly to the meal (fig 9A). At day 26, the glucose levels were similar to the levels at day 5, there were no significant differences between the dietary groups at day 26 (fig 9B).

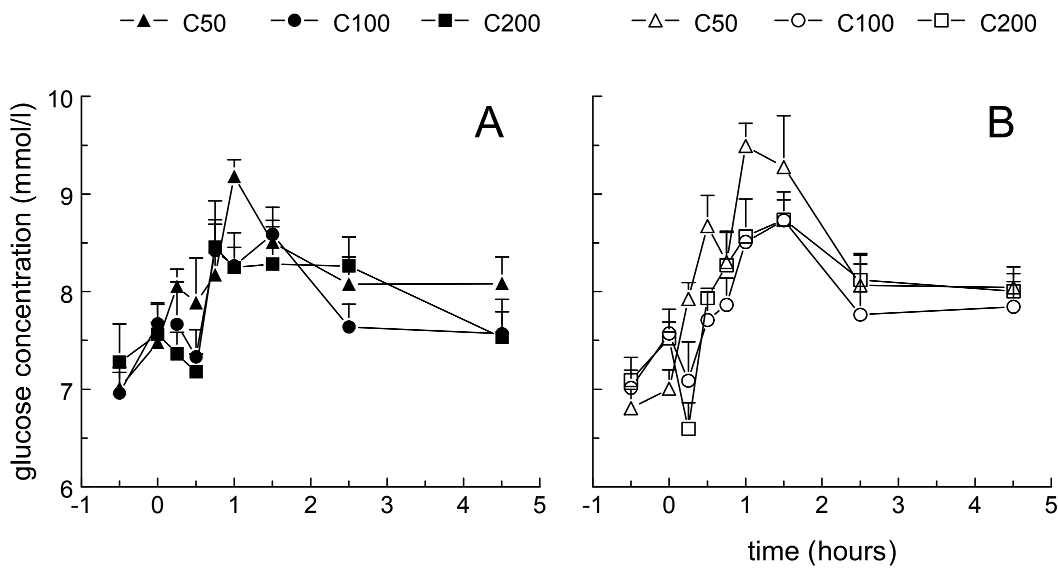


Figure 9. Plasma glucose levels of the group fed free amino acid diet with 50% methionine compared to the level of casein (C50), the group fed free amino acid diet with the casein amino acid pattern (C100), and the group fed free amino acid diet with 200% methionine compared to the level of casein (C200). Presented are the group averages + SEM (mmol/l). Panel A; short-term adaptation (day 5). Panel B; long-term adaptation (day 26).

Discussion

Body weight

Some studies have reported different growth rates for animals which received free amino acid diets compared to protein diets (Thorell & Lanner, 1973). In this experiment the body weight development of all dietary groups was similar as we experienced in earlier experiments (Nolles *et al.*, 2003).

Insulin

Interval effect

In both experiments, the RC group during the experiment was fed the same feed as was fed since weaning. This was done to monitor the possible effect of the adaptation period independent of dietary changes. Both experiments did not show significant differences between day 5 and 26 for any of the hormones or for blood glucose concentrations. Therefor the adaptation period as such had no effect on the hormonal responses to a meal. We studied hormonal responses to casein or casein based free amino acid diets on day 5 and day 26 of the adaptation period. Possible differences of the hormonal responses between these days must therefore be subscribed to the adaptation to the diets.

Dietary effects

When an animal is fed a novel meal, postprandial metabolism of amino acids from a free amino acid meal and a protein meal differs. Postprandial oxidative losses of feeding free amino acids are higher in comparison to those when amino acids are fed as components of proteins in a meal (Boirie *et al.*, 1997; Metges *et al.*, 2000). The utilization efficiency is negatively related to the appearance rate in the blood (Boirie *et al.*, 1997; Dangin *et al.*, 2001). It was therefore concluded that the differences between the utilization rates of free amino acids and protein in a meal are caused by the differences in appearance rate of the amino acids. Since size of the insulin response is also reported to be dependent on the appearance rate of the amino acids in the blood (Calbet & MacLean, 2002), For free amino acid diet a higher insulin response could be expected. However, for the animals fed the free amino acid diet diets, the insulin responses were similar to the casein group.
In both experiments, the insulin responses showed some differences at some time points. Due to the high variation those differences were not statistically significant and the physiological importance can be questioned. Insulin is known to react to absorbed glucose. It would be

expected that differences in insulin responses cause different blood glucose values. The values for insulin at day 5 at different time points are not correlated with values for blood glucose at corresponding time points.
Our previous studies showed that the oxidative losses decreased in time during adaptation to free amino acid diets (Nolles *et al.*, 2003). The insulin responses to the meal were similar at day 5 and 26. Adaptation to free amino acid diets is not mediated by the insulin response.
In our treatment glucose level in the meal was much higher than the amino acid level. So, the possible effects of different amino acid sources on insulin were masked by the glucose in the meal. Glucose causes a high insulin response in combination with the amino acids in the meal (Pallotta & Kennedy, 1968; van Loon *et al.*, 2000b). The different methionine levels in experiment 2 were not likely to cause different insulin responses since they were only a small part of the meal. In addition, especially the branched chain amino acids and arginine are known for their effect on insulin release and not methionine. Therefore, within a balanced meal the amino acids are probably not a dominating factor to determine the size of the insulin response.

Glucagon

Similar to insulin, the glucagon data showed no differences between diets, or between the measurements days. The responses of those individual hormones, both affect postprandial metabolism, but it is the insulin/glucagon quotient that determines the actual result. This insulin/glucagon quotient was similar for all dietary groups, so there were no differences between the measurement days.

Glucose

The blood glucose values can be an indicator for the appearance rate of dietary glucose and indirectly also for amino acids. However, no differences in blood glucose levels were observed. The glucose value increased even before the meal. This increase was possibly caused by increased activity in anticipation to the meal (Davidson & Stephan, 1999). The insulin response to a meal is well known to be also related to anticipation to the expected meal and secondly, it is a response to the absorption of nutrients.

Corticosterone

Corticosterone is known to be a catabolic hormone. Corticosterone reduces the effect of insulin on muscle glucose uptake, glycogen synthesis and proteolysis (Dardevet *et al.*, 1998; Perry *et al.*, 2003). This property makes the hormone suitable to regulate the utilization efficiency of meal-

derived amino acids. However, the postprandial corticosterone levels were similar in the different dietary groups. From this study we conclude that the corticosterone levels do not cause the postprandial differences between the oxidative losses of a free amino acid meal and a protein meal. In experiment 2, the corticosterone levels were not measured. Although we would not expect that corticosterone plays an important role in regulating the severity of the postprandial oxidative losses of methionine, it cannot be excluded.

Growth Hormone

Growth hormone is a well known stimulator of protein accretion. Improvements in protein status, either caused by increased digestibility, amino acid pattern or quantity, are accompanied by higher GH levels.

In rats, GH is released in a pulsatile manner. The growth hormone patterns of the animals varied within the dietary groups, both with respect to peak times and peak heights. Therefore, no differences between the groups could be detected.

General remarks

During the postprandial period, the oxidation rate of amino acids of a free amino acid meal was clearly higher than the oxidation rate of those of a protein meal, and the oxidative losses of methionine were affected by its abundance in the diet. This study was designed to elucidate the causes of these differences. The hormonal responses to the meals with the different amino acid sources did not differ. However, the physiological effects of the same hormonal responses on protein synthesis might be different due to the modulating effects of the source of ingested amino acids.

Thus leucine and arginine are known to stimulate protein synthesis, especially in combination with insulin. Therefore, it is hard to discriminate between metabolic and hormonal regulation. It seems well possible that the abundance of methionine triggers its own oxidation by substrate induction. Metabolic regulation may play a key role in amino acid metabolism, independent from endocrine effects.

Chapter 5

Adaptation to dietary amino acids, fed in free form, affects amino acid absorption in the rat intestine, assessed with the everted sac technique.

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Summary

In this study we investigated, with the everted sac method, the absorption rate of methionine or leucine in the small intestine of rats adapted to free amino acid diets for different periods of time. Male Wistar rats were adapted to a 21% free amino acid diet (FAA) with an amino acid pattern based on of casein. Before the start of the dietary treatments, rats were conditioned to housing condition and the feeding schedule. The adaptation period to the FAA diet was, 0 (non-adapted (N-ADA)), 5 (short-term adapted (ST-ADA)), or for, 26-33 days (long-term adaptated (LT-ADA)). The N-ADA animals were conditioned on a 21% casein diet. Within the N-ADA and the LT-ADA groups, the free amino acid diets were tested with 3 different methionine levels: 50%, 100% and 200% of the level present in casein. All diets were iso-nitrogenous and iso-caloric. The methionine absorption of the rats of the LT-ADA group was higher than that of the N-ADA group. Adaptation to 200% dietary methionine levels caused a significant lower leucine absorption compared to the 100%, and 50% group. There was no significant difference between the methionine absorption of the 50%, 100% and the 200% diets, but the absorption of methionine in the 50% group was enhanced in the distal part of the intestines. We concluded that in response to free amino acid diets, amino acid absorption was decreased to avoid toxic effects of high levels of indispensable amino acids.

Introduction

A general finding in amino acid requirement studies is the so-called 'Dietary protein paradox' that means higher amino acid supply with the feed, results in a lower utilization efficiency (Moundras *et al.*, 1993; Morens *et al.*, 2000; Dangin *et al.*, 2001; Morens *et al.*, 2001; Dangin *et al.*, 2002; Bos *et al.*, 2003; Morens *et al.*, 2003). Dietary amino acids are oxidized when the absorbed amount, exceeds the protein synthesis capacity. A high appearance rate in the blood and the tissues seems to be the causal factor. (Bos *et al.*, 2003) showed that the appearance rate of dietary amino acids in the blood after the meal is positively related to the oxidation rate of the amino acids.

The utilization efficiency of meals with free amino acids is equal or lower compared to meals with protein (Henry *et al.*, 1992; Officer *et al.*, 1997; Metges *et al.*, 2000; Daenzer *et al.*, 2001). However, the utilization efficiency of a free amino acid meal can improve in time (Nolles *et al.*, 2003). This suggests that metabolic and physiological adaptation mechanisms are induced by free amino acid diets.

The amino acid absorption rate by the intestine, contributes to the appearance rate in the blood at least partly. Amino acids are transported by both Na^+-dependent and Na^+-independent transporters and a small fraction of the amino acids passes the gut by simple diffusion (Mailliard *et al.*, 1995). Some transporters are very specific for a restricted number of amino acids, while other transporter systems mediate the transport of a wide range of amino acids.

There are different Na^+-dependent transporters for acidic basic and neutral amino acids. The acidic amino acids have a distinct Na^+ -independent transporter, but the basic and acidic amino acids do also share a Na^+ -independent transporter (Karasov *et al.*, 1986).

The intestinal amino acid transport systems are capable of adapting to changing levels of dietary amino acids (Jean *et al.*, 2001). Low protein diets do not affect the absorption of nonessential amino acids, but the transport of essential amino acids is stimulated (Karasov *et al.*, 1987; Schröder *et al.*, 2003). The intestinal amino acid transport capacity is up regulated by high protein meals. This applies for the non-esential amino acids but to less extent for the essential amino acids, that are toxic at high levels (Karasov *et al.*, 1987; Erickson *et al.*, 1995). Supplementation of protein diets with high levels of a single amino acid, leads to down regulation of the transport rate of that specific amino acid (Soriano-Garcia *et al.*, 1999). The intestine facilitates absorption of high protein meals, but limits amino acids acid absorption of essential amino acids to protects the body against toxic levels.

In the present study, we investigated amino acid absorption capacity of the small intestines of rats adapted for different periods (0, 5 or 26 days) to free amino acid diets. The period of 5 days was chosen since animals given a novel diet only eat small amounts of the diet during the first days. Day 5 is the first day all animals eat their meal completely, and some adaptation can already have taken place. Some adaptation is probably even needed to eat the whole free amino acid meal. At day 26 the animals are supposedly fully adapted to the diet.
The goal of this study was to test, to what extent the amino acid transport rate acts as an adaptational tool to reduce postprandial oxidative losses related to dietary free amino acids.

Materials and methods

Intestinal amino acid absorption is investigated in 3 separate everted sac experiments. With this method, the amino acids have to pass the luminal membrane and the basolateral membrane. Both passive diffusion and active transport is taken into account. The absorption of methionine and leucine is assessed with animals that have received a free amino diet for 0 days, 5 or 26 days. General design of the 3 experiments is given in Table 1. The feeding schedule and experimental procedures were similar and will be described below. This study was approved by the Wageningen University Animal Ethics committee.

Table 1. Overview of the experiments. Experiment 1; CAS is a group of animals that is preconditioned on rat chow with 21 % of casein as protein source. This group is not adapted to free amino acids (N-ADA). FAA is a group of animals, that is preconditioned on rat chow, with 21% free amino acids, with the pattern of casein. Experiment 2 and 3; 50%, 100% and 200% groups are preconditioned on a diet with 21% free amino acids with the pattern of casein with respectively 50%, 100% and 200% of the methionine present in casein.

	Animals	Amino acid	Adaptation	Diet	Tracer
exp 1	6	methionine	33 days (LT-ADA)	FAA	-
	6	methionine	0 days (N-ADA)	CAS	-
exp 2	7	methionine	5 days	50%	L-[1-14C]-methionine
	6	methionine	26 days	50%	L-[1-14C]-methionine
	7	methionine	26 days	100%	L-[1-14C]-methionine
	7	methionine	26 days	200%	L-[1-14C]-methionine
exp 3	7	leucine	5 days (ST-ADA)	50%	L-[1-14C]-leucine
	7	leucine	5 days	100%	L-[1-14C]-leucine
	7	leucine	5 days	200%	L-[1-14C]-leucine
	7	leucine	26 days (LT-ADA)	50%	L-[1-14C]-leucine
	7	leucine	26 days	100%	L-[1-14C]-leucine
	7	leucine	26 days	200%	L-[1-14C]-leucine

Animals

Divided over 3 studies, 81 male Wistar rats (300 g) were utilized. The animals were obtained from Harlan (Horst; The Netherlands). Initially the rats, were group housed in macrolon cages (55 x 33 x 18 cm), bedded with sawdust with a light schedule of 8 hours dark (red light, 4-5 Lux) and 16 hours light. The dark period was from 9.00 till 17.00 hours. The daily feed intake (20 g) was divided over two distinct meals, provided at 9.00 and 16.30 hours (Teklad Global 18% Protein rodent diet; Harlan, The Netherlands).
The animals were given 30 minutes to eat each meal, which was sufficient time to finish the meal. The habituation to the new environment, light schedule and feeding regime persisted for at least two weeks for all animals. After this habituation period, the animals received the experimental feed.

Table 2. Complete composition of the experimental diets, expressed as g/100 g feed (w/w dry matter). Left column; all non-amino acid components. Middle and right column; The amino acid components for the FAA and the CAS diets of experiment 1, and the 100% methionine group of experiment 2 and 3. The 50% and the 200% group of experiment 2 and 3 consisted half, and double methionine levels as given in this table respectively.

Feed composition					
Glucose	63.9	Alanine	0.61	Lysine	1.81
Cellulose	5.0	Arginine	0.71	Methionine	0.59
Soybean oil	5.0	Aspartic acid	1.39	Phenylalanine	0.99
$CaCO_3$	1.24	Cysteine	0.053	Proline	1.79
$NaH_2PO_4.2H_2O$	0.34	Glutamic acid	4.64	Serine	1.18
$MgCO_3$	0.14	Glycine	0.36	Threonine	0.88
KCl	0.11	Histidine	0.57	Thryptophan	0.25
KH_2PO_4	1.05	Isoleucine	1.01	Tyrosine	1.09
Vit/Min. mix	2.2	Leucine	1.87	Valine	1.24
Total	790	Total amino acids 21.0 g			

In experiment 1; the rats had been adapted for 33 days to a diet (Table 2) in which 21% amino

acids were given as casein (CAS) or as free amino acids (FAA). At day 33, in vitro methionine absorption by the small intestine was assessed with the everted sac method (Singh *et al.*, 1996). In experiment 2 and 3; Each group had been conditioned for 5 or 26 days to 1 of 3 diets with 21% casein supplied as free amino acids, with 50, 100 or 200% of the methionine level of casein.

Everted sac method

Overnight fasted animals were put in a box flushed with O_2/CO_2 mixture. As soon as the animals lost consciousness the oxygen administration was stopped. As the animals stopped breathing, the small intestine was carefully removed. Intestinal content was removed and the inside of the intestine was rinsed twice with ringer solution.

In experiment 1, the intestine was divided into four pieces of equal length. Part 1 being the most proximal, and part 4 being the most distal part. These four parts were each cut in half. From these four pairs of intestine parts, one of the pair was used for absorption measurement the other was used as control (see below). The pairs were assigned in a random manner. The intestine parts were turned inside out and closed on one side to form an everted intestinal sac. The other end of the sac was attached to a small plastic tube (diameter 1.5 mm). The intestine was suspended in the cylinder with ringer solution.

In experiment 2 and 3, the intestines were divided in four pieces of equal length. Each part was tied with cotton thread on one side to form an everted intestinal sac. The other end of the sac was tied to a small plastic tube (diameter 1.5 mm). Since experiment 2 and 3 were measured using ^{14}C labeled leucine and methionine a control was not necessary.

Experimental procedure

A 100 ml glass cylinder was filled with 100 ml Ringer solution with 1 g/l glucose. The solution was carbogygenated (95% O_2, 5%CO_2) prior and during the measurements. The cylinder was placed in a water bath of 37 °C.

Experiment 1; As substrate for amino acid absorption, 1.145 g/l methionine was added to the cylinder. 1 ml Ringer was injected through the tube into each everted sac. Thereafter one of each pair of the everted intestinal sacs was suspended in a cylinder with added methionine, the other part served as a blank and was placed in a cylinder with Ringer solution without added methionine. The open ends of the everted sacs were kept just above the Ringer solution. The everted sacs were incubated for a period of 90 minutes.

Experiment 2 and 3; The everted sacs were suspended in a 100 ml glass cylinder similar to experiment 1, but filled with 80 ml Ringer solution.
Different absorption substrates were added to this solution for the subsequent experiments; 2 ml Ringer solution (including 1 g/l glucose) was injected through the plastic tube on the open side into the everted sac.
In experiment 2; 1.145 g/l ^{12}C-methionine plus L-[1-^{14}C]-methionine (tracee free) as tracer (1 μCi), specific activity 52 mCi/mmol (ARC, USA, St Louis).
In experiment 3; 1.00 g/l ^{12}C-leucine labeled with so called "tracee free" L-[1-^{14}C]-leucine 1.0 mCi/mmol, Amersham pharmacia Biotech).

Analysis

After 90 minutes of incubation, the outside of the everted sacs was first rinsed with saline, and the content within the everted sac was collected.
Experiment 1; Millipore water was added to all samples to obtain a total volume of 10 ml. The fluid was deproteinated with Sulfosalic acid method and analyzed for methionine content.
Experiment 2 and 3; 15 ml scintillation liquid was added to the sample (Ultima Gold, Packard Bioscience) to a total volume of approximately 20 ml. Thereafter it was analyzed by a liquid scintillation analyzer (Tri-carb 1900 CA, Packard).

Statistics

Data are presented as mg/90 min + SE. Statistical analysis were performed using the computer program SPSS 11.5.0 (2002). Students t-test was used to test for significant differences between groups. When multiple comparisons are made, an analysis of variance was performed (ANOVA), bonferroni test was used as posthoc test. Differences were considered significant when $P < 0.05$.

Results

Experiment 1; N-ADA compared to LT-ADA

Results of this experiment are presented in figure 1. The intestinal methionine absorption, as measured "ex vivo" with the everted sac technique, was significantly higher (P = 0.014) for the animals fed casein diet and not adapted to free amino acid diet (N-ADA), than for the animals that have been fed free amino acid diets for more than 3 weeks (LT-ADA). In all 4 parts of the intestines, the methionine absorption in the N-ADA group is significant higher (P = 0.025) than that in the respective intestinal part of the LT-ADA group (fig 1).

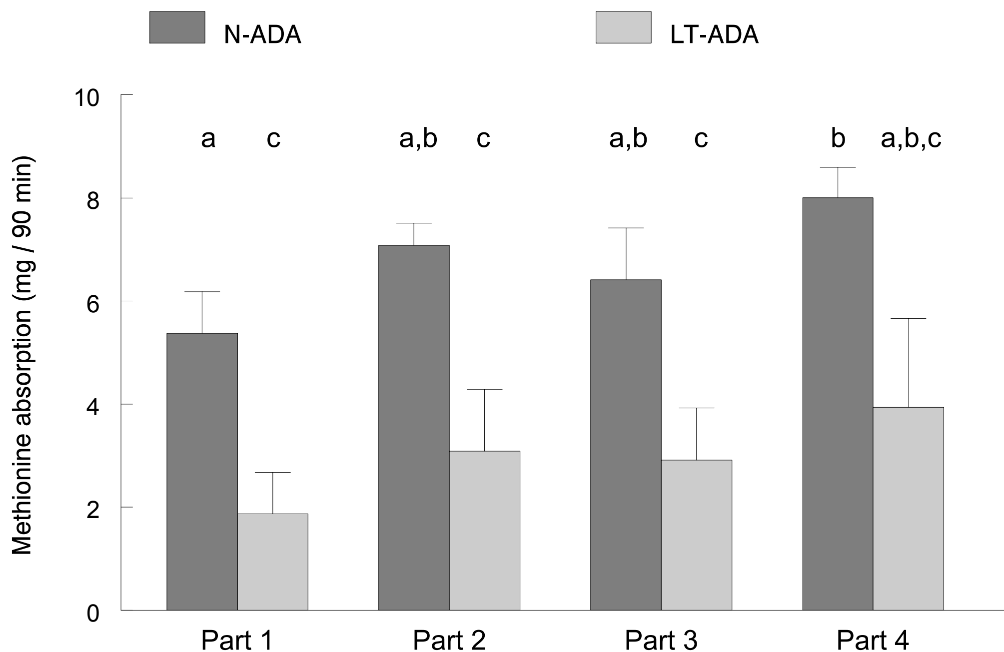

Figure 1. Small intestinal methionine transport estimated with inverted sac technique. Compared are intestines of rats adapted to free amino acid diets for three weeks ore more (LT-ADA) with those of rats not adapted to free amino acid diets (N-ADA) prior to the measurements. The intestine is divided in 4 equal parts; part 1 is the most proximal part, part 4 is the most distal part. The experimental groups indicated with "CAS" are adapted to a diet with 21% casein as sole amino acid source. The free amino acid groups are adapted to a diet with 21% free amino acids. Groups not sharing the same letter, differ significantly.

The intestinal methionine absorption over the whole intestine (the 4 intestinal parts cumulated) was 26.9 ± 1.1 mg per 90 min for the N-ADA rats, and 11.3 ± 4.6 mg methionine for the LT-ADA rats (P =0.01). The methionine absorption rate tends to increase from proximal to the more distal parts of the intestine. In both the LT-ADA and the N-ADA group, a significant higher methionine absorption was found in part 4 compared to part 1 (P = 0.03).

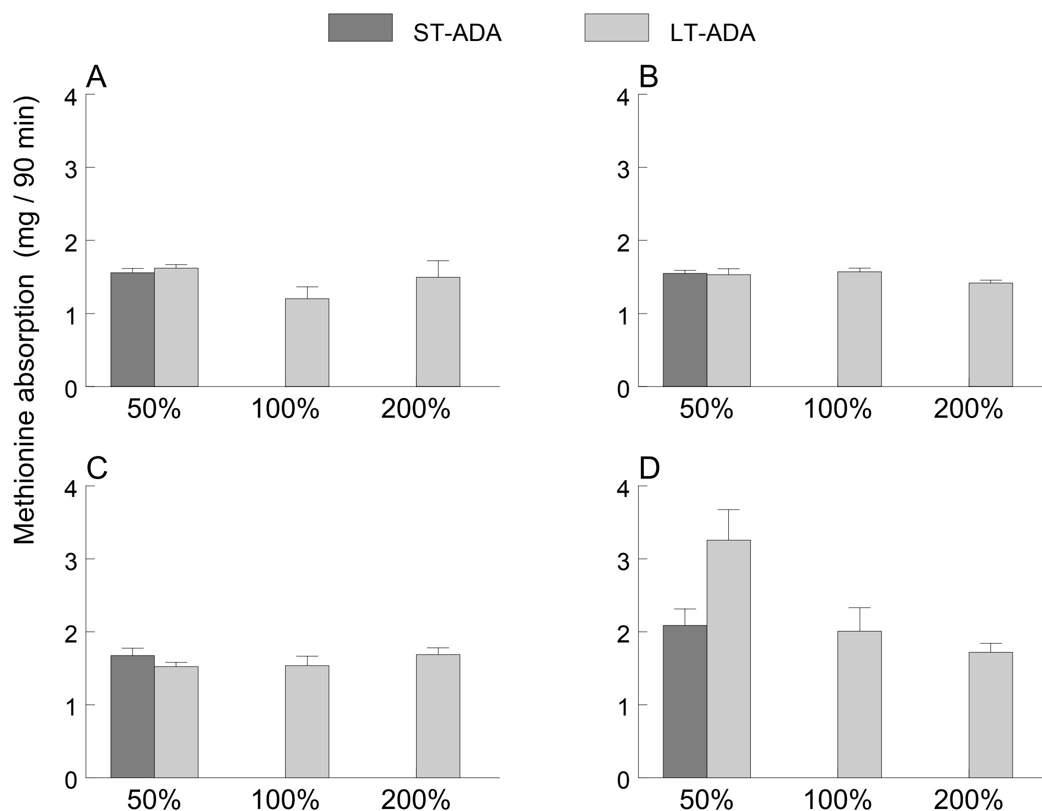

Figure 2. Methionine transport (mg/90 min +SE) over the small intestine wall of animals with different dietary preconditioning, measured in vitro with everted sac technique. 50%, 100% and 200% refer to the level of methionine in the diet compared to the level in casein. Dark bars are animals that had for 26 days adaptation to Methionine (LT-ADA), bright bars represent animals adapted for 5 days to the diet (ST-ADA). Data are expressed as methionine absorbed from the everted sac (mg/90 min + SEM). Panel A; part 1 of the intestine (proximal). Panel B; intestine part 2. Panel C; intestine part 3. Panel D; intestine part 4 (distal).

Experiment 2; Methionine absorption of rats adapted to 50 100, or 200% methionine

The methionine recovery, in the everted sacs from the intestine parts 1, 2 and 3, was approximately 1.6 mg (fig 2). This was similar for all dietary groups. The most distal part of the intestine showed some differences in absorption. The methionine recovery in the 4th part of the intestine of the ST-ADA 50% group, was higher than the recovery in part 2 (P = 0.048). The LT-ADA 50% group showed the highest value (3.3 ± 0.4 mg)in the 4th part. This was significantly higher than part 4 of the 200% group (P = 0.03) and it was also higher than the recovery in part 1, 2 and 3 of the LT-ADA 50% group (P = 0.02).

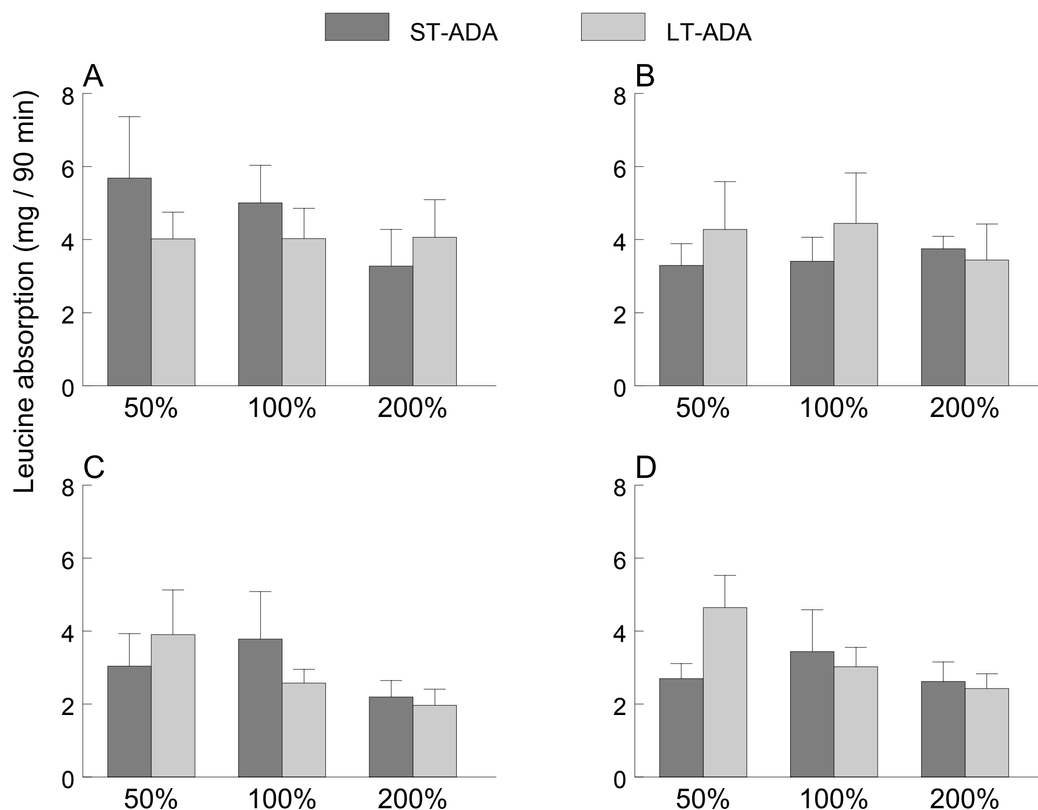

Figure 3. Leucine absorption (mg/90 min + SE) over the small intestine wall measured in vitro with everted sac technique. Rats are adapted to a casein based free amino acid diets with all amino acids and either 50% (50%), 100% (100%) or 200% (200%) of the methionine present in casein. Panel A; part 1 of the intestine (proximal). Panel B; intestine part 2. Panel C; intestine part 3. Panel D; intestine part 4 (distal).

Experiment 3; Leucine absorption of rats adapted to 50 100, or 200% methionine

This experiment was designed to test to what extent different dietary methionine levels affect the absorption of leucine at different adaptation periods (fig 3).

Methionine levels did affect leucine transport (ANOVA; P = 0.03). The 200% group showed the lowest leucine absorption (11.0 ± 1.5 mg leucine, over the whole intestine). This was significantly lower (bonferroni; P = 0.04) than the 100% diet group (14.6 ± 2.3 mg leucine), but not significantly lower (bonferroni; P = 0.07) than the 50% group (15.4 ± 2.1).

The leucine absorption in intestinal part 1, 2, 3 and 4 was similar (ANOVA; P = 0.184). There were no significant differences between the leucine absorption of intestines from ST-ADA rats compared to LT-ADA rats. Although the 50% group showed a tendency that with adaptation to the low methionine level, leucine transport in the most distal part of the intestine increased.

DISCUSSION

The background of this study was to determine whether amino acid absorption is used to modulate the utilization efficiency of dietary amino acids. To get more insight about the mechanisms that regulate the utilization efficiency, different models can be used. We varied the duration of the adaptation to a diet. The everted sac method was used to measure the amino acid absorption rate. The utilization efficiency depends both on the characteristics of the feed, and the physiological handling of the feed by the animal. The characteristics of the feed that are important are digestibility, digestion rate, the amino acid level and the amino acid pattern.

From literature (Metges *et al.*, 2000; Daenzer *et al.*, 2001) it is known that free amino acid diets are oxidized at a higher level than protein based diets especially in the postprandial phase. This is mainly caused by higher appearance rates of amino acids in the blood after free amino acid feeds (Bos *et al.*, 2003). (Nolles *et al.*, 2003) showed that rats can adapt to free amino acid diets, resulting in that the postprandial oxidative losses of amino acids decrease in time. Our hypothesis was that adaptation of amino acid transport over the gastro-intestinal tract into the blood is one of the mechanisms involved in this adaptation.

It could be expected that the first goal of the intestine is to maximize amino acid absorption. Both low and high protein diet have been shown to increase the amino acid absorption capacity. In the case of high protein meals, amino acid absorption was up regulated to facilitate the absorption of the large protein part of the meal (Karasov *et al.*, 1987; Erickson *et al.*, 1995; Schröder *et al.*, 2003). Low protein diets have also been shown to increase the amino acid absorption capacity (Schröder *et al.*, 2003) As the authors stated, this adaptation is probably a mechanism to minimize amino acid losses with the feces. It is well known that amino acids in order to be of value for the animal, need to be absorbed in the small intestine. So amino acids that enter the large intestine will be broken down by microbes, or will be excreted.

Free amino acids can be absorbed quickly. Thus free amino acid diets have a high appearance rate and produce high amino acid levels.

Since high levels of especially the indispensable amino acids can be toxic (for review (Harper *et al.*, 1970)); diets that cause high appearance rates result in high postprandial oxidative losses. The amino acid utilization is limited by protein synthesis capacity but also by the appearance levels at which amino acids are toxic. As already shown for methionine (Soriano-Garcia *et al.*, 1999), the intestine reacts with down-regulation of amino acid absorption.

In the first experiment, we compared the intestinal amino acids absorption capacity in rats that were adapted to a free amino acid diet or to a protein diet. The latter are thus not adapted to

dietary free amino acids. The methionine absorption rate of long term adapted (LT-ADA) animals decreased to 50% of the level of the non-adapted (N-ADA) group. This is in-line with previous observations (Karasov *et al.*, 1987). He found also substantial differences in amino acid absorption between animals fed protein and animals fed diets where the protein is replaced by free amino acids. However the animals came from different batches and he suggested that the differences are caused by differences between the batches. In our opinion, animals fed free amino acid diets decreased methionine absorption, because this can be a powerful tool to decrease the high amino acid appearance rate occurring after ingesting a free amino acid diet. This is one of the adaptational mechanisms to decrease the relative oxidative losses accompanying free amino acid diets.
Within experiment 2 and 3, we compared LT-ADA (26 d) with ST-ADA (5 d). Between those groups no significant differences in absorption were observed. This indicates that at least a large part of the adaptation has already taken place before day 5 (ST-ADA).

In addition to the differences between the diets in experiment 1, it was also shown that the intestinal amino acid absorption was clearly different between the different parts of the intestine. The AA absorption in part 4 was significantly higher than that of part 1. This difference found in experiment 1 was not replicated in experiment 2 and 3. The diets of the 100% group in experiments 2 and 3 were similar to experiment 1, but experiment 3 did not show any difference between the intestinal parts and experiment 3 (compared to experiment 1), showed a reversed tendency for leucine transport. Intestinal leucine absorption was even lower towards the distal parts of the intestine. It seems that it is not a general property regarding which part of the intestine is the most important for amino acid transport.
Leucine and methionine are both neutral amino acids. Due to the characteristic they share the same amino acid transporters. Both these amino acids have access to amino acid transporters that are accessible to the other (Karasov *et al.*, 1987). In this perspective, we compared the results of experiment 2 and 3. The dietary effects on methionine and leucine absorption would be expected to be similar although methionine is varied and not leucine. High and low amino acid levels can both increase and decrease methionine absorption. In experiment 3, the leucine absorption was decreased in the group fed a diet with increased methionine level. The 50% group showed an increased absorption, although this was only seen in the distal part of the intestine. This increase seems to be an adaptation to minimize methionine losses in and out the large intestine. Since dietary methionine can be absorbed very quickly when fed as free amino acid. Increased methionine absorption in the 4th part of the intestine can hardly have any effect on the efficiency

of the dietary methionine absorption, but it can probably reduce the losses of endogenous methionine. Although it has no effect on the utilization efficiency of dietary amino acids, it still can reduce postprandial amino acid losses.
Conclusion; The overall strategy is not only to maximize amino acid absorption but more likely to maximize utilization. In case of free amino acid diets, this leads to decreased rate of absorption speed.

Chapter 6

General Discussion

General Discussion

Introduction

The most simple way to study amino acid utilization is to compare the nitrogen intake with the nitrogen excretion via urine and fecal matter. This method only gives an average value of the nitrogen-balance over the period studied, but neglects the amino acid kinetics over the day (Waterlow, 1999a). At least two phases are important; the postprandial phase and the postabsorptive phase. In the postprandial phase dietary proteins are digested and absorbed and the dietary amino acids are incorporated in body protein, or they are oxidized (Harris *et al.*, 2004). Once oxidized, the amino acids are lost for protein metabolism. In a N-equilibrium situation high utilization efficiency in the postprandial phase is accompanied by higher postabsorptive amino acid oxidation. Amino acids, that are oxidized during the protein turnover, should not be considered to be real losses, since they contribute to the physiological functions of the protein turnover (Millward, 1998). In this respect, the postprandial oxidative losses actually determine the nutritional status (Madani *et al.*, 2000).

There are two important questions we wanted to answer in this thesis. First; How does the kinetics of dietary amino acids affect the postprandial oxidative amino acid losses? Secondly; What are the adaptational mechanisms to adjust the amino acid appearance rate postprandially to the protein synthesis capacity?

The amino acid kinetics was manipulated and studied in several ways. In chapter 2 we studied the metabolic handling of 2 dietary amino acid sources, each with a different appearance rate. In this study rats are adapted to diets with protein as the only amino acid source, or to diets with only free amino acids. We also adapted rats to a 50% mixture of those diets. Measurements were carried out at day 5 and day 26 of the adaptation period.

These experiments made clear that at day 5 (short term adaptation) the oxidative losses are higher for dietary free amino acids than for dietary protein (chapter 2). This result is supported by the observation that the recovery of the dietary amino acids in the tissues is lower after ingesting free amino acid diets than after ingesting protein diets. To study the metabolic handling more in detail, in chapter 3, we assessed the postprandial oxidative losses of free amino acid diets, with different methionine contents. Both the oxidative losses of methionine and those of leucine were measured. Leucine was used as a tracer for nearly all other amino acids. Increase of methionine levels caused higher oxidative losses of methionine, but the oxidative losses of leucine were not affected by this (chapter 3).

At day 26 (long term adaptation) the oxidative losses of the dietary free amino acids had decreased and the recovery of amino acids in the tissues had increased. We concluded from this study, assuming 5 days is short-term, that dietary free amino acid diets are subjected to higher oxidative losses than protein diets, but on the longer term (day 26), the body is able to adapt to dietary free amino acids. For supplementation of proteins deficient in one or more amino acids this means that, at short notice, more free amino acids have to be supplemented than the amino acid requirement would suggest (Reeves *et al.*, 1993). On the longer term animals are able to adapt to free amino acid diets and differences between free and protein feeding diminishes.
In chapters 4 and 5, studies were conducted to get more insight in the adaptational mechanisms that reduce the oxidative losses. To study whether the adaptation to free amino acids is mediated by a change in postprandial hormonal responses, we have measured insulin, glucagon, GH, and corticosterone in the same settings and the same diets as the experiments in chapter 2 and 3. This has been reported in chapter 4. To study whether the main adaptational mechanism to free amino acid diets is to reduce the appearance rate of the dietary amino acids, we measured intestinal methionine and leucine uptake of rats adapted to free amino acid diets for different periods. This was done with the everted sac method.

Amino acid appearance rate

The utilization efficiency of dietary amino acids, especially correlates with the appearance rate of those amino acids in the blood (Bos *et al.*, 2003). Since the free amino acid pool in the blood is rather constant and small (Millward *et al.*, 1996), compared to the dietary intake of amino acids and the free amino acid level in the blood is rather, the dietary amino acids can only stay in the blood for a short time, Thus they will be transported into the tissues immediately. Therefore, the appearance rate into the blood affects the appearance rate in the tissues to a large extent.
The appearance rate is very important because amino acids can only be stored as protein. High amino acid levels can be toxic and in the situation, when the appearance rate exceeds the protein synthesis rate, the excessive amino acids are degraded (oxidative losses). The protein synthesis capacity relative to the appearance rate is of crucial importance for the utilization efficiency. Protein synthesis is mainly stimulated by substrate induction (Millward *et al.*, 1996). Logically protein synthesis is activated by lower amino acid levels, than the enzymes that oxidize the amino acids (Harris *et al.*, 2004). But amino acid oxidation dominates at situations with high amino acid levels. The utilization efficiency is therefore higher with low appearance rates, than with dietary amino acids with a high appearance rate. The appearance rate can be affected by several factors. These factors include dietary amino acid sources (Hall *et al.*, 2003) and metabolic handling of

the dietary amino acids (Calbet & MacLean, 2002; Hall *et al.*, 2003).
In general there are only 3 ways to decrease oxidative losses related to high appearance rates. The body should either increase the protein synthesis capacity to the level of the amino acid appearance rate, or the body should decrease the appearance rate of endogenous amino acids (derived from the protein turn over), or the body should reduce the appearance rate of the dietary amino acids to the blood, The amino acid oxidation rate cannot decrease without one of those mechanisms unless the amino acid levels will rise and this will have toxic side effects. For those adaptational processes, the question rises how this adaptation is regulated.

Role of protein synthesis

The experiments with the different amino acid sources in the feed (chapter 2) gave important information about the nature of the adaptation mechanisms. The oxidative losses for dietary free amino acids were similar in the diet with free amino acids as single amino acid source and the mixed diet with both free amino acids as protein. The oxidative losses for intrinsically labelled protein were similar in the pure diet with protein as the only amino acid source and in the mixed diet. The experiment showed clearly that the different amino acid sources were metabolized strictly independent. The presence of protein in the diet, did not affect the metabolic handling of the dietary free amino acids and vice versa.
After adaptation, the oxidative losses of the dietary free amino acids had diminished to the level of that from protein. While the oxidative losses of amino acids derived from protein were not affected. This strong distinction between the amino acids sources, is very clear in the 100% groups with only free amino acids or protein in the feed. But this distinction is as clear in the 50% groups with both amino acid sources present in the same feed.
The reduced oxidative losses, observed in the 100% free amino acid group could possibly be achieved by both an increased protein synthesis capacity, as by a reduced appearance rate (Millward *et al.*, 1996). The experiment with both amino acid sources in one diet, allows us to discriminate between those adaptational mechanisms. The oxidative losses of the free amino acid part decrease but the oxidative losses of the protein part of the meal remain similar at day 5 and day 26 of the adaptation period. In my opinion this shows that a change in protein synthesis capacity in the tissues, is not the primal site of adaptation. Since, an increased protein synthesis rate would affect both sources of dietary amino acids in the 50% groups with the mixed diets.

Hormonal responses; effects on protein synthesis

Postprandial regulation involves several responses of hormones that are known to affect amino

acid metabolism. I have chosen to measure hormones which are known to affect protein synthesis and protein degradation. In this respect insulin is the most well known hormone.
Insulin has a positive effect on postprandial amino acid utilization. This effect is mainly due to an inhibiting effect on protein degradation (Gibson *et al.*, 1996; Fereday *et al.*, 1998).
Whereas, the amino acid availability is postprandially the main stimulator of protein synthesis (Millward *et al.*, 1996). The effect of insulin on protein synthesis is less pronounced and even negative effects of insulin on protein synthesis have been reported (Tessari *et al.*, 1996; Ang *et al.*, 2000; Tessari *et al.*, 2003).
But insulin enhances the stimulating effect of branched chain amino acids on protein synthesis. Especially leucine is known for its stimulating effect (Anthony *et al.*, 2000b, Lynch, 2002 #350). Insulin does only enhance protein synthesis when the amino acid supply is sufficient. When not enough amino acids are available, insulin only reduces protein breakdown, without enhancing protein synthesis (Balage *et al.*, 2001).

Protein degradation

The inhibition of protein degradation is the most powerful tool to decrease the nett amino acid appearance rate. The decrease of protein degradation that occurs normally in response to a meal is mediated both by substrate induction and by the insulin response. Therefore an increased insulin response could be well expected in the animals, that were long-term adapted to the free amino acid diet. But this increased insulin response was not observed. We realize that the protein degradation rate has to be measured to exclude a difference between the protein degradation rate after a free amino acid-based diet and after a protein-based diet. However, we did not measure the postprandial protein degradation rate. Although we cannot exclude that protein degradation is decreased as adaptational mechanism to reduce postprandial oxidative losses, we expect that mainly other processes were changed in the adaptational process.

Intestinal amino acid transport

To reduce the oxidative losses related to free amino acid diets, the most logic mechanisms would be those, that reduce the appearance rate of the dietary amino acids (Rivest *et al.*, 2000). We investigated, wether, at the intestinal level, some adaptational changes have occurred (chapter 5), with the everted sac method.
The first experiment described in chapter 5 had very clear results. Intestinal leucine resorption rate was significantly lower in the intestines of long-term adapted rats (26 days to free amino acids) compared to non-adapted rats (not adapted to free amino acids). We concluded that the

intestinal amino acid absorption rate was lowered as a result of an adaptational mechanism to reduce the appearance rate of the dietary amino acids.
Intestinal enterocytes begin as immature crypt cells. Over a period of 2-4 days the enterocytes differentiate and migrate up the crypt villus axis. In response to changed diets the enterocytes can alter their nutrient transport during maturation. This may include amino acid and peptide transporters.
Due to the time it takes the enterocytes to mature, it takes the intestine at least 2-4 days to adapt to the free amino acids diets. Probably closer to 4 days than 2 days for the whole intestine. When the rats were first served their experimental diet, they did hardly eat any food during the first few days. So the adaptational process did probable not start before day 2. We chose to do all our experiments at day 5 and day 26, since day 5 was the first day the rats eat their whole meal.
Day 5 of the adaptation period could be a critical day in the adaptational process. It is well possible that the properties of the amino acid transport systems were still changing over the day.

The everted sac experiments did not show any differences in absorption rates between day 5 and day 26. It seems that the adaptation on intestinal level has already taken place at day 5. That is quite remarkable, since we saw striking differences between those days with respect to the postprandial oxidative losses of free amino acid diets.
The everted sac experiments were conducted 5-6 after the start of the meal, during the postabsorptive phase. This suggests, that the everted sac probably does not represent the situation during the postprandial phase at day 5. But because of the changes of the amino acid absorption between non-adapted rats and long-term adapted rats, we conclude that adaptation of the intestinal amino acid absorption is a most likely the tool to reduce the postprandial oxidative amino acid losses.

50% diets; possible explanation

The most striking result presented in this thesis, in our opinion, is the fact that protein and free amino acids do not interact at the level of postprandial oxidation. At day 5 protein does not affect the metabolic handling of the free amino acid fraction and the free amino acids do not affect the protein fraction. The most precarious about this is, that from 1.5 hour after a meal, label originating from both the protein as from the free amino acid fraction is recovered in the breath. That means, that both free amino acids and protein derived amino acids are being absorbed, transported to the tissues and incorporated in body protein or oxidized. Apart from intestinal tissues, the other tissues (e.g. liver) cannot discriminate between those amino acids derived from

different sources. Therefore differentiation between those amino acid sources must already have been made in the intestines or in the stomach.

After long-term adaptation, the oxidative losses for the free amino acid fraction of the 50% diets has decreased to the same extend as the losses from the 100% diet and the adaptation process did not affect the oxidative losses of the protein fraction. From the results of the everted sac experiments we concluded that the adaptation to free amino acid diets included a decreased amino acid absorption rate by the intestines.

The digestion products of protein are small peptides and amino acids (Webb, 1990), but the majority of the digestion products are small peptides, for review (Ray *et al.*, 2002; Daniel, 2004; Steffansen *et al.*, 2004). Those small peptides are absorbed by several peptide transporters (e.g. PEPT1) (Daniel, 2004). Peptide transporters are localized in the apical membrane, but no peptide transporter has been found in the basolateral membrane. Therefore, most peptides are hydrolysed within the enterocytes (Steffansen *et al.*, 2005). The resulting amino acids are than metabolised within the enterocytes, or transported by amino acid transporters in the basolateral membrane into the circulation.

This differential absorption mechanism could explain why a decreased intestinal amino acid absorption rate does not affect the appearance rate of protein derived amino acids. As stated before, the amino acid absorption rate determines the appearance rate and the appearance rate determines the oxidative losses. Both protein derived amino acids and dietary free amino acids are transported by the same transporters over the basolateral membrane. Since the adaptation mechanisms to the mixed diets do not affect the protein derived amino acids, I hypothesize that especially the amino acid transporters in the apical membrane are involved in the adaptation process. Furthermore down regulation of apical membrane amino acid transport does protect the both intestinal tissues and the other tissues against high amino acid levels.

The intestine has probably an important role in determining the postprandial amino acid losses. The intestine does not only absorb the amino acids, it is also a tissue with a high activity in amino acid metabolism. The intestine preferably uses dietary amino acids to synthesise proteins (for e.g. enzymes transporters etc)(Stoll *et al.*, 1998a; Stoll *et al.*, 2000). The intestine plays an important role in amino acid catabolism (Stoll *et al.*, 1998b; Wu, 1998; Goudoever van *et al.*, 2000; Lobley *et al.*, 2003). These characteristics make the intestine a key determent in amino acid utilization. The intestine is the only organ that actually can discriminate between dietary free amino acids and protein derived dietary amino acids. The increased oxidative losses observed for free amino acid diets are probably due to the intestine itself.

Final conclusions

1. At first exposure, the postprandial oxidative losses of dietary amino acids are higher for ingested dietary free amino acids, than for dietary protein.

2. Free amino acids and protein, present in the same meal, are metabolized in an independent way.

3. Increased dietary methionine levels are partly stored (feed driven), although the oxidative losses are relatively higher.

4. The methionine level in free amino acid diets hardly affects the postprandial oxidative losses or storage of the other dietary amino acids.

5. Rats adapt to free amino acid diets and decrease the postprandial losses.

6. Adaptational responses of rats to free amino acid diets are not mediated by insulin, glucagon or GH.

7. Adaptational responses of rats to free amino acid diets are mediated by reduced appearance rate of dietary amino acids from the digestive tract.

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Appendixes

Nederlandse samenvatting

Dankwoord

Publications

Curriculum Vitae

Samenvatting

Eiwitturnover

Eiwitturnover is het proces waarbij de eiwitten in het lichaam voortdurend onderhevig zijn aan synthese en afbraak. Deze turnover van lichaamseiwitten is cruciaal voor het repareren van beschadigde eiwitten en voor het kunnen inspelen op de behoefte aan lichaamseiwitten met functionele eigenschappen die optimaal passen bij fysiologische omstandigheden. Tijdens de turnover van eiwitten kan ongeveer 80% van de aminozuren worden hergebruikt. Ongeveer 20% wordt afgebroken en doorgesluisd naar het energiemetabolisme. Dit verlies aan aminozuren moet worden aangevuld door aminozuren uit de voeding. De aminozuren die vrijkomen bij de afbraak van lichaamseiwitten komen eerst in de intracellulaire pool van vrije aminozuren en kunnen via het bloed worden uitgewisseld met andere weefsels.

Postprandiale fase

De postprandiale fase is de fase direct na een maaltijd waarin het voedsel wordt verteerd en geresorbeerd. De eiwitten uit de voeding worden afgebroken tot kleine peptiden en vrije aminozuren. Deze peptiden en aminozuren worden door de darmcellen opgenomen. De peptiden worden in de darmcellen vrijwel volledig verder afgebroken tot losse aminozuren. Deze aminozuren kunnen door de darmcel zelf worden gemetaboliseerd of worden afgegeven aan het bloed. Via het bloed worden de aminozuren dan naar de weefsels getransporteerd.
Aminozuren kunnen in het lichaam vrijwel niet in vrije vorm worden opgeslagen. Hoge concentraties vrije aminozuren zijn niet alleen toxisch ze zijn ook osmotisch actief en veroorzaken zouden daardoor invloed hebben op het volume van de vrije aminozuurpool. Grote hoeveelheden aminozuren kunnen alleen worden opgeslagen in de vorm van eiwit. Dit betekent dat het patroon van de opgeslagen aminozuren altijd overeenkomt met dat van de lichaamseiwitten. De meeste lichaamseiwitten zijn samengesteld uit 20 aminozuren maar de relatieve hoeveelheid kan verschillen. Om aminozuren op te slaan als eiwit moet dus het complete patroon van het te vormen eiwit aanwezig zijn. Om de aminozuren uit de voeding tijdens de postprandiale fase op te kunnen slaan als eiwit moet de eiwitsynthesecapaciteit toereikend zijn. Na de maaltijd wordt de capaciteit voor netto eiwitsynthese verhoogd door de afbraak van lichaamseiwit tijdelijk te verlagen. Hierdoor wordt de eiwitturnover tijdelijk onderdrukt en wordt de beschikbare eiwitsynthesecapaciteit gebruikt om de aminozuren uit de maaltijd op te slaan als eiwit.
Problemen kunnen optreden als de concentratie vrije aminozuur in het lichaam stijgt wanneer de opname van aminozuren uit de darm hoger is dan de capaciteit van de eiwitsynthese om ze op

te slaan als lichaamseiwit. Om de aminozuurconcentratie op een aanvaardbaar niveau te houden stimuleert een toename in van de aminozuurconcentratie de oxidatie van aminozuren. Wanneer op deze wijze een substantieel deel van de aminozuren uit de voeding wordt geoxideerd heeft dit een negatief effect op hun metabole benutting van het voedingseiwit. Daarom is het belangrijk te weten hoe de metabole benutting van aminozuren uit de voeding kan worden geoptimaliseerd. In het algemeen wordt het stikstofverlies tijdens de postabsorptieve fase gecompenseerd door een stikstofwinst tijdens de postprandiale fase. Zodoende kan het lichaam in stikstofbalans blijven bij verschillende niveaus van eiwitinname. Daarbij zorgt een hoge eiwitinname voor een hogere positieve stikstofbalans tijdens de postprandiale fase, dan een lage eiwitinname, mits de aminozuren goed opgeslagen kunnen worden. Dit geeft het lichaam meer mogelijkheden om te reageren op wisselende behoeftes. Daarom is een hoge eiwitinname en benutting gunstig voor de adaptatiecapaciteit van het lichaam.

Benuttingsefficiëntie van de aminozuren uit de maaltijd

Het doel van dit proefschrift is een beter begrip te krijgen van de processen die de benuttingsefficiëntie van de aminozuren uit de maaltijd beïnvloeden.

Het onderzoek dat in dit proefschrift wordt beschreven is speciaal gericht op de snelheid waarmee de aminozuren na een maaltijd verschijnen in de vrije aminozuur pool van het lichaam. Het is gebleken dat de verschijningssnelheid van de aminozuren in deze pool invloed heeft op de postprandiale oxidatie van aminozuren. De verschijningssnelheid beïnvloedt daardoor de benuttingsefficiëntie van aminozuren uit de voeding voor fysiologische doeleinden. In dit proefschrift is bestudeerd in hoeverre het lichaam kan omgaan met een dieet waarvan de aminozuren zeer snel door het lichaam worden opgenomen. Tevens is geprobeerd meer duidelijkheid te verschaffen over de mechanismen hierbij betrokken zijn.

Verschijningssnelheid in de vrije aminozuurpool van het lichaam.

Allereerst is vastgesteld wat de metabole consequenties als aminozuren uit de voeding snel na een maaltijd in het bloed worden opgenomen. Deze studies zijn uitgevoerd met een [$^{13}CO_2$]-ademtest in modelstudies bij zowel de rat als de mens.

Hierbij zijn diëten gebruikt waarvan het aminozuurdeel bestond of uit ei-eiwit of uit vrije aminozuren. In het dieet met ei-eiwit werd voor de experimentele maaltijd gebruik gemaakt van ei-eiwit waarin [1-^{13}C]-leucine was ingebouwd. In het dieet met vrije aminozuren werd het leucine vervangen door [1-^{13}C]-leucine. Tevens werden deze twee diëten tijdens de studie mengsel aangeboden (1:1). Tijdens de experimentele maaltijd werd in dit mengsel of het

aminozuur of het eiwit gedeelte gelabeld met [1-^{13}C]-leucine. De metingen werden gedaan op dag 5 en op dag 26 na overschakeling op het dieet.
Op dag 5 bleken de postprandiale oxidatie van leucine uit het dieet met vrije aminozuren significant hoger dan voor het dieet met ei-eiwit. Voor het gemengde dieet werden dezelfde oxidatieve verliezen gemeten afhankelijk van de gebruikte labeling.
Uit de resultaten van dit onderzoek werd geconcludeerd dat de aminozuren afkomstig van ei-eiwit en van vrije aminozuren onafhankelijk van elkaar worden gemetaboliseerd, zelfs als ze via dezelfde maaltijd worden ingenomen. De resultaten van de modelstudies met de mens waren vergelijkbaar met de studies bij de rat.
De verschillen tussen de diëten met vrije en van ei-eiwit afkomstige aminozuren waren grotendeels verdwenen na een adaptatieperiode van 26 dagen, de verschillen in oxidatieve verliezen waren vrijwel verdwenen. De ratten waren kennelijk in staat zich aan te passen aan de diëten met vrije aminozuren.
In de tweede studie is bestudeerd in hoeverre de verandering van het methioninegehalte in het voer (50, 100 of 200% t.o.v. caseïne) invloed heeft op de opslag in lichaamseiwit. Verandering van het methioninegehalte in het voer zal invloed hebben op de verschijningssnelheid in de vrije aminozuurpool. Deze verschijningssnelheid heeft weer invloed op de benuttingsefficiëntie van de aminozuren uit de maaltijd. Een bijkomend probleem is dat in principe alleen complete patronen (corresponderend met de samenstelling van bepaalde lichaamseiwitten) kunnen worden opgeslagen. Bij een stijging van het aanbod van methionine in het dieet kan ook een verhoogde oxidatie van het methionine worden verwacht.
Voor de diëten met het hoogste aanbod van methionine werd de laagste benuttingsefficiëntie gevonden. Echter werd, in absolute zin, wel meer methionine vastgelegd. Na lange termijn adaptatie (3 weken) was retentie van vrije aminozuren, gemeten via methionine, in alle dieetgroepen verhoogd.
Geconcludeerd werd dat de postprandiale retentie van aminozuren, in ieder geval voor een deel, gedreven wordt door de aminozuursamenstelling van aangeboden dieet.

Factoren die invloed hebben op de benuttingsefficiëntie.

In de derde studie werd bekeken of de postprandiale benuttingsefficiënte hormonaal wordt gereguleerd. Uit onze studie bleek dat de verschillen in oxidatieve verliezen tussen diëten met vrije aminozuren of ei-eiwit, niet afhankelijk zijn van de gecombineerde werking van insuline, glucagon, corticosteron en groeihormoon. Het postprandiale catabolisme van aminozuren wordt waarschijnlijk door andere factoren bepaald, zoals het actuele aanbod. Zoals al eerder is gezegd,

heeft de verschijningssnelheid van aminozuren uit de maaltijd in de vrije aminozuurpool een duidelijk effect op de postprandiale benuttingsefficiëntie.

In de vierde studie is onderzocht of de absorptiesnelheid van aminozuren door de darm verandert na het verstrekken met diëten met vrije aminozuren. Ratten kregen een dieet met vrije aminozuren gedurende 0 (niet geadapteerd), 5 (korte termijn adaptatie) of 26-30 dagen (lange termijn adaptatie). De absorptie van methionine in lange termijn geadapteerde ratten was duidelijk verlaagd ten opzichte van niet geadapteerde ratten. Geconcludeerd werd dat de snelheid van aminozuurabsorptie een cruciale rol speelt bij het minimaliseren van de postprandiale oxidatieve verliezen van aminozuren.

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Curriculum Vitae

Jelmer Auke Nolles werd geboren op 24 April 1974 te Leeuwarden. Hij groeide op in de Trynwâlden. Hij haalde zijn VWO diploma in juni 1993 aan de Christelijke Scholengemeenschap Oostergo te Dokkum en begon zijn studie Biologie aan de Rijks Universiteit Groningen. In 1999 behaalde hij zijn doctoraal examen met de als afstudeerrichting gedrags- en neurowetenschappen. Van januari 2000 tot januari 2006 was Jelmer werkzaam als Assistent in Opleiding (AIO) aan de leerstoelgroep Fysiologie van Mens en Dier van de Wageningen Universiteit. De resultaten hiervan zijn in dit proefschrift beschreven.

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Full papers

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J.A. Nolles, I.G.S. Peeters, R.E. Koopmanschap, M.W.A. Verstegen and V.V.A.M. Schreurs Metabolic Adaptation to Free Amino Acid Diets with Different Methionine Levels in Rats. *To be submitted*

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