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Amino acid utilization and isotope discrimination of amino nitrogen in nitrogen metabolism of rat liver *in vivo*

Aminosäurenverwertung und Isotopendiskriminierung des Aminostickstoffs beim In-vivo-Stickstoffstoffwechsel der Rattenleber

Summary Urea and plasma protein differ in natural ^{15}N abundance up to 10‰. The origin of this difference is the branched nitrogen metabolism in the liver. One main branch is the protein synthesis pathway, the other the urea synthesis pathway. By this branching ^{15}N of precursor amino acids is depleted in urea while it is enriched in protein. With the ^{15}N abundance of precursor amino acids, which may be taken from jejunum tissue, utilization of amino acids in liver metabolism can be calculated from isotope discrimination in either pathway.

This was investigated by feeding different proteins to rats. When feeding high quality protein (whey protein) utilization of amino acids in liver metabolism at requirement intake was better than at zero protein intake (> 85% vs. 70%). From this we conclude that the pattern of amino acids available from the metabolic pool at zero protein intake is characterized by an imbalance. This endogenous imbalance can be complemented by exogenous dietary amino acids so that nitrogen excretion may even be smaller than the so-called „obligatory" losses of intakes not exceeding requirement. Thus, the quality of dietary protein is reflected not only by N balance. It also may be quantified by analysis of isotope discrimination in nitrogen metabolism of the liver. In addition, the quality of amino acid pattern available from the metabolic pool is indicated by this method.

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Zusammenfassung Zwischen Harnstoff und Plasmaprotein besteht ein natürlicher Unterschied der ^{15}N-Häufigkeit von bis zu 10‰. Die Ursache hierfür ist der verzweigte Stickstoff-Metabolismus in der Leber. Ein Hauptzweig ist die Proteinsynthese, im zweiten Hauptzweig wird der Aminostickstoff zur Harnstoff-Synthese verwendet. ^{15}N der Vorläuferaminosäuren wird hierbei, abhängig von den Umsatzraten, im Harnstoff abgereichert und im Protein angereichert. Unter Hinzuziehung der ^{15}N-Häufigkeit der Vorläuferaminosäuren, die sich aus Jejunum-Gewebe bestimmen läßt, kann aus der Isotopendiskriminierung in den beiden Stoffwechselzweigen die Utilisierung der Aminosäuren in der Leber berechnet werden.

Dies wurde an Ratten nach Fütterung mit verschiedenen Proteinen untersucht. Es zeigte sich, daß bei Verzehr von hochwertigen Proteinen die Utilisierung der Aminosäuren im Stoffwechsel der Leber bei bedarfsgerechter Fütterung besser war (Molkenprotein: > 85%) als bei Proteinkarenz (70%). Das bedeutet, daß das Muster der aus dem metabolischen Pool verfügbaren Aminosäuren bei Proteinkarenz eine Imbalanz aufweist. Durch exogene Aminosäuren aus der Nahrung kann diese endogene Imbalanz offenbar komplementierend ausgeglichen werden, so daß die Stickstoffausscheidung bei nicht bedarfsüberschreitendem Verzehr sogar unter den Werten der sogenannten „obligaten" oxidativen Verluste liegen kann. Die Qualität

von Nahrungsproteinen spiegelt sich also nicht nur in den N-Bilanzen wider. Sie wird auch durch Bestimmung der Utilisierung der Aminosäuren im Stoffwechsel der Leber mit Hilfe der Analyse der Isotopendiskriminierung quantifizierbar. Zusätzlich wird mit dieser Methode eine Aussage über die Qualität des Musters der metabolisch verfügbaren Aminosäuren gewonnen.

Key words Protein quality – endogenous imbalance – ^{15}N – natural abundance – isotope discrimination

Schlüsselwörter Proteinqualität – endogene Imbalanz -^{15}N – natürliche Häufigkeit – Isotopendiskriminierung

Introduction

Utilization of amino acids may be measured by tracer techniques, applying amino acids labeled in the carbon skeleton with stable ^{13}C or the radioisotope ^{14}C, respectively. For the more general analysis of nitrogen metabolism the amino group of single amino acids or whole dietary protein may be enriched by ^{15}N. In this case the fate of heavy nitrogen is followed. Although more or less different results are obtained with different tracer amino acids [1] and although it is known that isotope effects do exist, it is assumed that the labeled tracer behaves exactly like the non-labeled substance.

We do the opposite and make use of the different behavior of the two naturally occurring stable isotopes of nitrogen. We study liver nitrogen metabolism by analyzing isotope discrimination at natural abundance of nitrogen isotopes [2, 3, 4].

Amino acid utilization denotes that fraction of amino acids which is not oxidized. In a first approximation amino acid utilization may be seen as the result of a branched pathway (Fig. 1). Precursor amino acids (A) lead to protein (P_1) on the one hand and to products of amino acid catabolism on the other hand. Amino nitrogen in this latter branch mainly is incorporated into urea (P_2).

Generally, an incomplete extent of a reaction results in discrimination of isotope pattern of those atoms which are directly involved in the reaction due to first order isotope effects [5]. This may be found even more in reactions of branched pathways. Primarily, this is caused by kinetic isotope discrimination and is strictly linked to the extent of turnover of the precursor by either branch [4, 5, 6, 7]. If one of the pathways would utilize 100% precursor, no overall isotope discrimination would be found between precursor and product, because all isotopes are transferred to the product. The only and generally fulfilled preconditions for isotope discrimination in branched pathways are that both branches are active so that neither pathway utilizes all precursor and the overall isotope effects in either branch are different [4]. In the case of nitrogen the light isotope ^{14}N is generally preferred to the heavy isotope by some per cent [7]. In the end product of that branch with larger overall isotope effect ^{15}N is depleted. In amino acid metabolism this is the catabolic branch leading to urea (Fig.1).

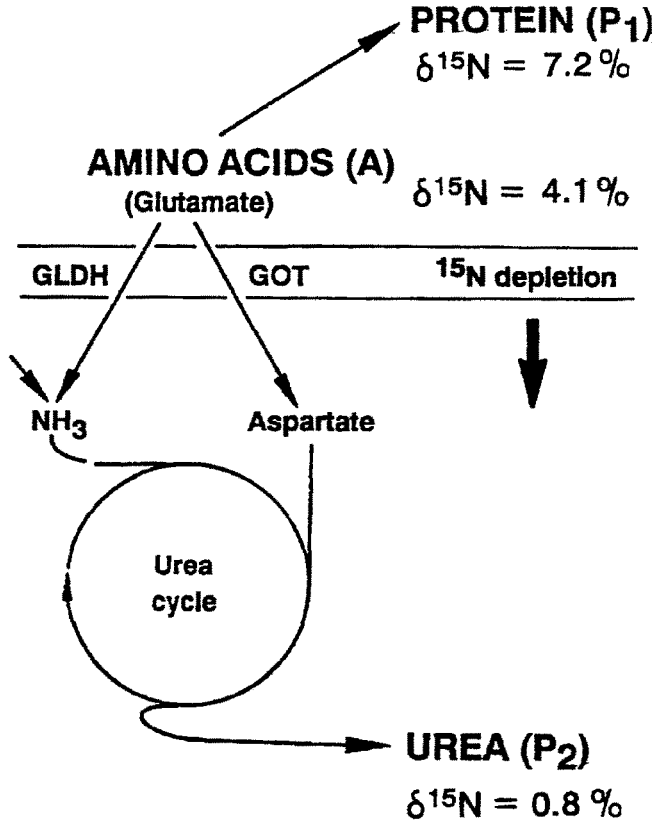


Fig. 1 Branched pathway of amino acids (A) as the common precursor of protein (P_1) and urea (P_2) in nitrogen metabolism of the liver. The $\delta^{15}N$ values are arbitrary figures illustrating the magnitude of isotope discrimination.

Transamination or the oxidative process of deamination affects directly the amino nitrogen and, therefore, isotope effects are significant [6, 7] and must be much larger than in the protein synthesis branch. As a result ^{15}N is depleted in urea and ammonia formed in the liver, while ^{15}N in proteins like serum albumin, which also is synthesized in the liver, is always enriched, if compared with the amino group of the common precursor, i. e., the amino acids of the metabolic pool. This phenomenon we termed "isotope ratio disproportionation" [2]. The overall result becomes apparent in main products of nitrogen metabolism of the liver (serum proteins and urea) which

differ in natural ^{15}N abundance by about 4 to 10‰ (per mill) (2). This difference is large if compared with the accuracy of isotope ratio mass spectrometers equipped with a changeover inlet systems. Such differences of ^{15}N abundance in the amino acids of different tissues have been known for 30 years (8).

^{15}N enrichment of liver protein depends on the ratio of protein-to-urea synthesis rate. The more protein is synthesized relative to urea, the less the protein is enriched while urea under these conditions is depleted more compared with the precursor (and vice versa) (4). Nitrogen isotope discrimination can be neglected in protein synthesis, because protein synthesis is an irreversible process with no rate limiting step characterized by first order isotope effects of amino nitrogen. This means that the higher the fraction of the precursor amino acids is metabolized to protein, the more similar is the isotope pattern of protein to that of the precursor amino acids. This means also that protein, which is synthesized in a metabolic compartment without significant amino acid oxidation, reflects the ^{15}N abundance of the precursor amino acids from the metabolic pool. From basic rules describing isotope ratio disproportionation it was derived that it is possible to quantify nitrogen metabolism of the liver in monogastric animals and man (2, 3). In this paper we describe measurement and calculation of amino acid utilization from the metabolic pool in nitrogen metabolism of the liver by this technique.

Materials and methods

Nitrogen isotope discrimination in nitrogen metabolism of the liver was analyzed in male Wistar rats. Animals were fed semisynthetic diets containing one of the following proteins in multi-level balance experiments without energy restriction: casein, casein + 142 mg methionine per g nitrogen, whey protein, wheat gluten, soy protein. In the diet, protein plus starch together amounted to 76.4% (w/w). Sucrose was 8.5%, soybean oil 5.0%, cellulose 5.0%, minerals and trace elements were 4.2%, and vitamins 0.9%. Minerals, trace elements, and vitamins were adapted to the requirements of rats and were purchased from Merck, Darmstadt, Germany. The sum of protein plus corn starch content was kept constant in all diets to make them isoenergetic.

Male Wistar rats (Harlan-Winkelmann GmbH, Borchen, Germany) of initially 50 to 100 g body mass (BM) were fed ad libitum a diet containing the test protein (Fig. 2). Different protein content in the diet (w/w) were chosen for the various test proteins: casein 14%, casein + methionine 9%, whey protein 9%, soy protein 20%, wheat gluten 25%. After having reached 200 g BM the animals were put into metabolic cages as described by Eggum (10) and fed for adaptation with the same diet for further 3 days. During the following 3 days, still on the same diet, a nitrogen balance was carried out to check the response homogeneity of the animals. After this balance period three animals each were grouped for different diets containing increasing amounts of the respective test protein.

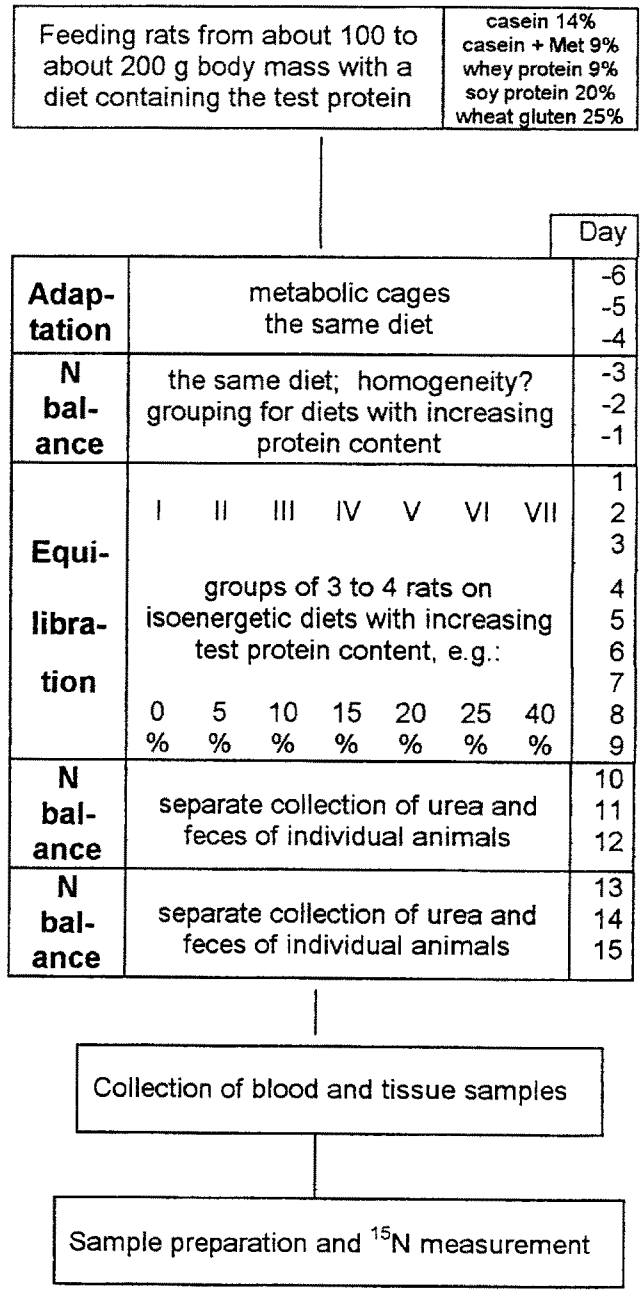


Fig. 2 Experimental design for measurement of nitrogen isotope ratio disproportionation dependent on intake of dietary protein. Protein content of the diets during equilibration and the second and third nitrogen balance were stepwise increased for the diet groups. Casein: 0, 5, 7.5, 10, 14, 20, 30%; whey protein: 0, 3, 5, 7, 9, 15, 30%; soy protein: 0, 5, 7.5, 10, 14, 20, 40%; wheat gluten: 0, 5, 10, 15, 25, 40%; casein + methionine (3 series): 0, 5, 10, 20, 40%; 3, 5, 6, 7, 8.5, 10, 13%; 0, 5, 7.5, 10, 15, 20, 40%.

After 9 days equilibration on the different diets two further 3-days nitrogen balances were performed. After the last balance period (after day 15) animals were sacrificed and blood and samples of different tissues were taken. The experimental design is summarized in Fig. 2. The gradation of the protein content of the diets was roughly adapted to the steepness of the dose response to the single dietary protein. This means that larger steps were chosen for wheat gluten and soy protein, respectively, while smaller ones at lower intake of the milk proteins.

^{15}N abundance was measured with a delta E mass spectrometer (Finnigan MAT, Bremen, Germany) which was coupled with an elemental analyzer (Heraeus, Hanau, Germany) via a trapping box interface. The delta notation ($\delta^{15}N$) is used expressing the relative ^{15}N abundance as per mill (‰) deviation from atmospheric nitrogen. The accuracy (external precision) was less than ± 0.1‰.

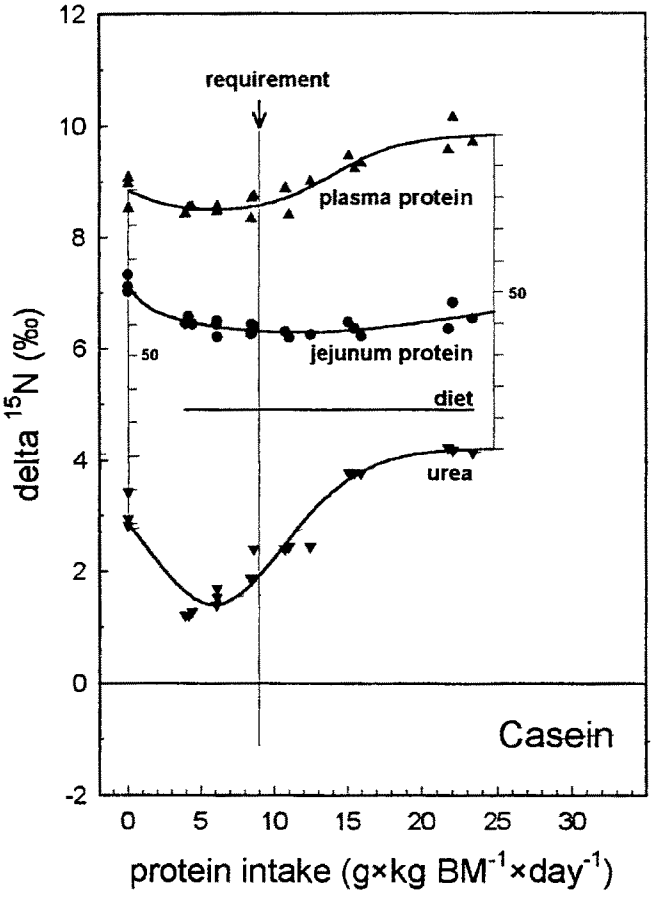
$\delta^{15}N$ values of plasma protein, of protein of the small intestine, and of excreted urea were evaluated for each animal. The observed effects are described presenting individual data points. Curves in the figures were calculated by non-linear regression analysis. Rates for amino acid utilization were compared using Student's t-test. Differences were considered significant at $p < 0.05$.

Results

The magnitude of the isotope ratio disproportionation of nitrogen in urea and serum albumin, which are both products of liver nitrogen metabolism, depended on the type of dietary protein as well as on protein intake level. This is demonstrated in Figs. 3 to 7.

In principle the data sets are similar in so far that in all cases serum protein produced in the liver always exhibited the highest $\delta^{15}N$ values. For urea the lowest values were always found, while $\delta^{15}N$ values of jejunum protein which was considered as an indicator of ^{15}N abundance of the precursor amino acids were somewhere between both and did not depend directly on $\delta^{15}N$ of the diet.

Natural ^{15}N abundances changed significantly dependent on protein intake which was most dramatic for urea nitrogen in the case of methionine-supplemented casein (Fig. 4). From requirement intake of 5 g/kgBM/day to intake levels of 15 g/kgBM/day $\delta^{15}N$ of urea changed from 0‰ to 5‰. At requirement intake $\delta^{15}N$ of urea exhibited a significant minimum value. A minimum value was also observed in case of the other dietary proteins, but this was less significant and (except soy protein) at less than requirement intake. Requirement intake was defined from nitrogen balance data and indicates that protein intake level at which values for nitrogen retention reached a plateau. In the figures requirement intake is indicated by a vertical line.



Figs. 3 to 7 Natural ^{15}N abundance of plasma protein, jejunum protein, and urea of rats after equilibration with diets containing different proteins (see inserts) at various protein intake levels.

On the abscissa the protein intake in g per kg body mass and day is given. The vertical arrow and line indicates the minimum requirement intake for optimum growth. These data were derived from nitrogen balance data of the animals and indicate the protein intake level at which maximum nitrogen retention was achieved.

On the ordinate the natural ^{15}N abundance using the delta notation referenced to atmospheric nitrogen is given. The straight horizontal line denotes the delta ^{15}N value of the respective dietary protein. The upper points are the values for plasma protein dependent on protein intake. The lower points are the values for urea, while in the middle the data of jejunum protein are given. The curves are trend curves and were derived from non-linear regression analysis, but do not consider a distinct physiological model.

100% scales are shown left and right for each set of curves. In theory the difference between urea and serum protein can be taken as a 100% scale cut into two intercepts by the precursor value which is assumed to be equal to the jejunum protein. The upper intercept corresponds to the urea synthesis rate and the lower to the protein synthesis rate in the liver, both as fraction of unity or 100%, respectively (4).

Large changes were also observed feeding casein (Fig. 3) or whey protein (Fig. 5), while moderate changes occurred feeding soy protein (Fig. 6) and wheat gluten (Fig. 7).

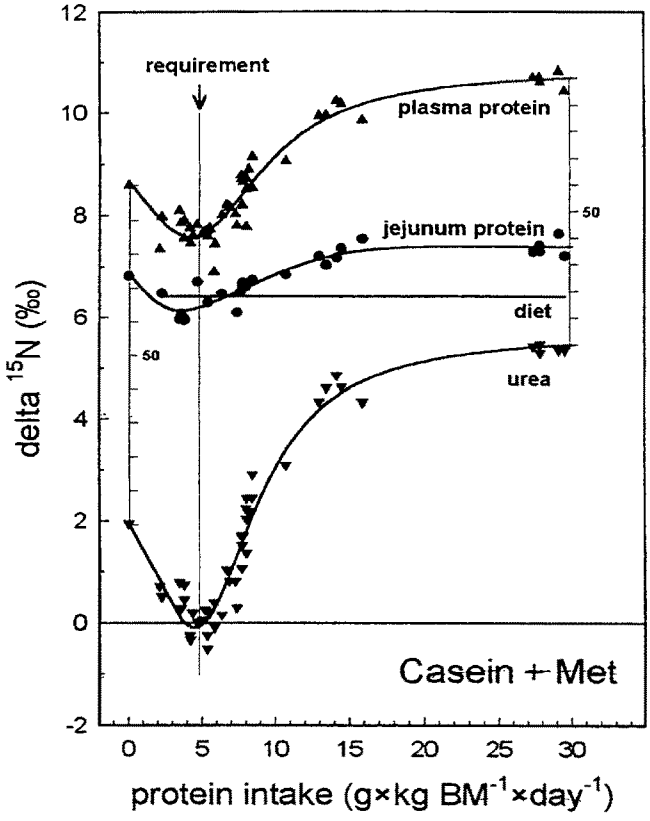


Fig. 4

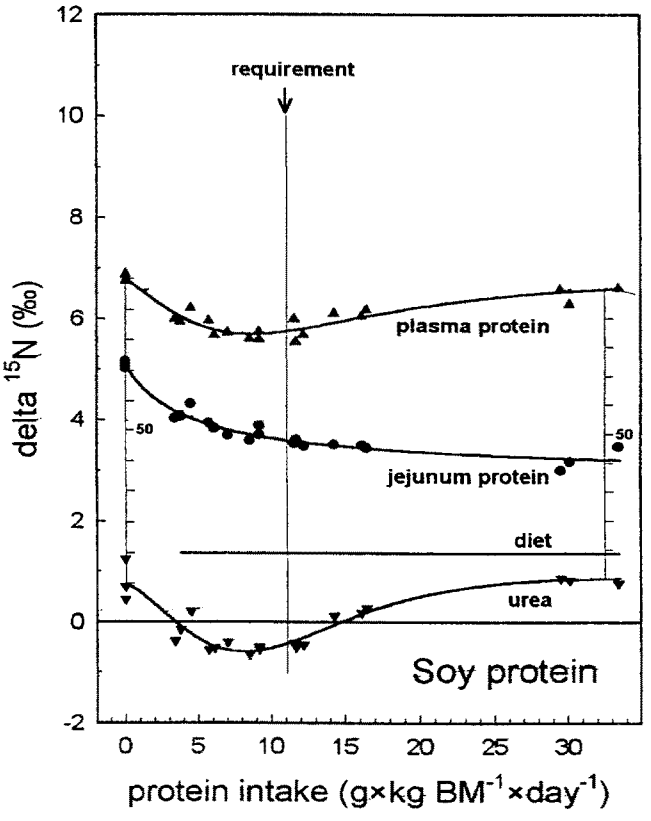


Fig. 6

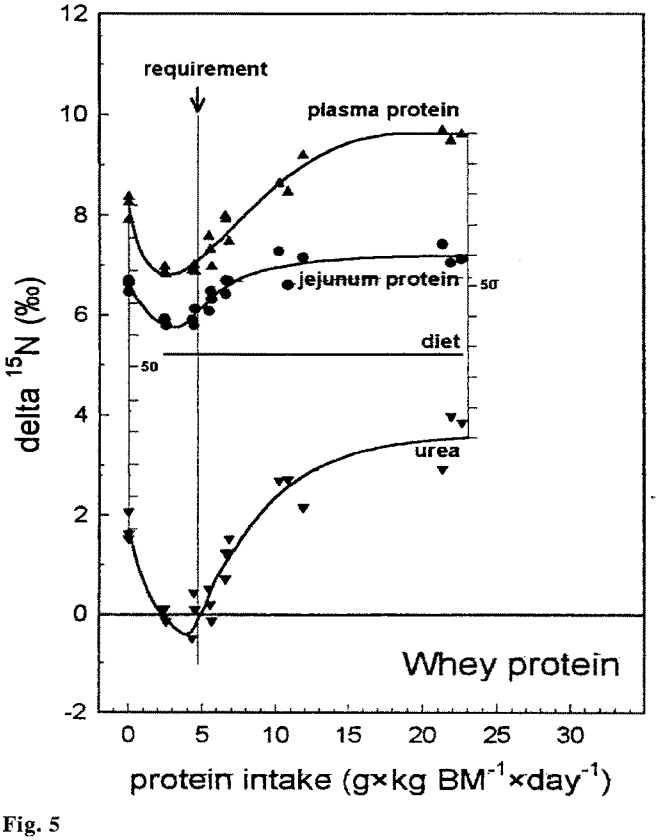


Fig. 5

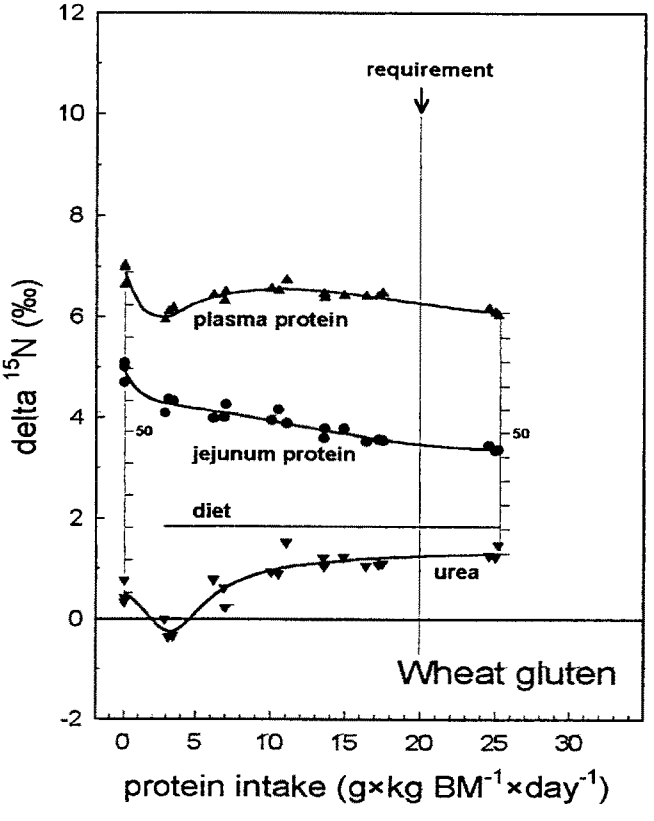


Fig. 7

The difference between the $\delta^{15}N$ values of plasma protein and urea at a distinct protein intake, which is the isotope ratio disproportionation and may be taken as 100%, is divided by the $\delta^{15}N$ value of the according jejunum protein into two intercepts. This is shown in the figures by the scales left and right of the data sets. At any protein intake level the upper intercept between the curves of serum and jejunum protein corresponds to the fractional urea synthesis rate, while the lower intercept between jejunum protein and urea curve corresponds to the fractional protein synthesis rate. These intercepts indicate low urea synthesis at low protein intake and high urea synthesis rate at highest protein intake if compared to protein synthesis rate. When feeding casein, soy protein or wheat gluten, respectively, the ratios of these intercepts were not significantly different at highest protein intake level. Despite the balance data (not shown) at high intake were similar also for whey protein, this ratio was increased, indicating a higher protein synthesis rate ($p < 0.05$) than in the case of the other proteins. For the whole range of protein intake these ratios are shown by the curves in Fig. 8.

Well known differences were observed for protein requirement intake:
9 g casein/kgBM/day were needed, while on methionine supplementation it was only 5 g/kgBM/day. In the case of whey protein 5 g/kgBM/day were also needed, for soy protein it was 10 g/kgBM/day, and for wheat gluten more than 20 g/kgBM/day.

Feeding of whey protein, casein, and especially methionine supplemented casein revealed an isotope ratio disproportionation (difference between $\delta^{15}N$ of plasma protein and $\delta^{15}N$ of urea) which was maximum around 5g protein/kg/BM/day. This maximum was not found when feeding the rats soy protein or wheat gluten, respectively.

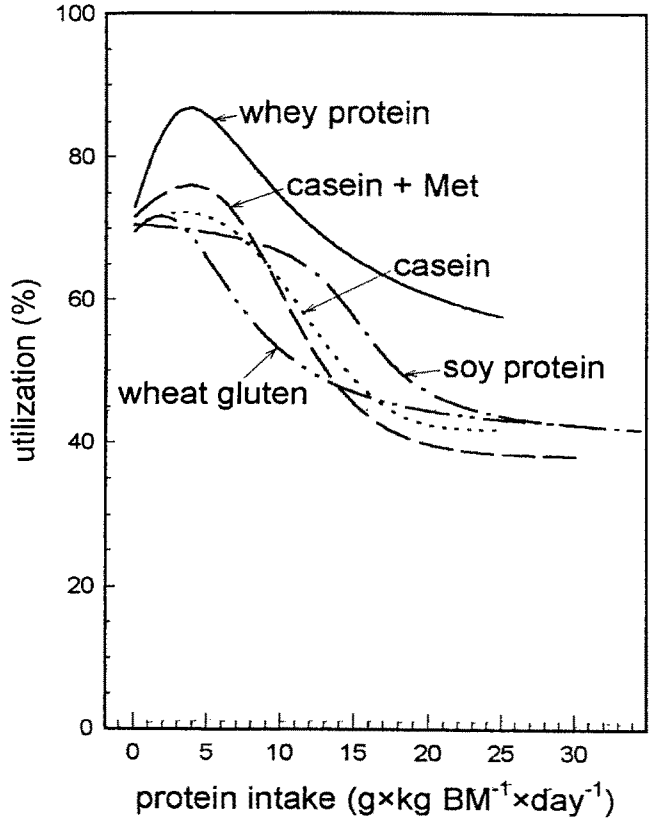


Fig. 8 Utilization of amino acids from the metabolic pool in nitrogen metabolism of the liver as calculated from isotope ratio disproportionation dependent on the intake of different proteins.

Discussion

Non-statistical distributions of isotopes in products of metabolism are well-known. In the 60's Gaebler et al. (8) found different $\delta^{15}N$ abundances in different amino acids from distinct tissues of the same organism. Later it was demonstrated that a large number of biochemical reactions, catalyzed by enzymes, are accompanied by significant isotope effects (summarized in [9]). In the case of nitrogen metabolism the pathways of amino acid catabolism play a key role for major discrimination of heavy and light nitrogen. Concerning this, the presented data are discussed referring to mechanistic rather than simply empirical aspects. The largest isotope effects in nitrogen metabolism occur, if amino nitrogen itself is affected as it is in the case of transamination. Transamination from glutamate to oxalacetate (glutamic-oxalacetatic transaminase, GOT) forming aspartate results in 10‰ difference of ^{15}N abundance between formed aspartate and remaining glutamate (6). Isotope discrimination in ammonia formation on oxidative desamination by glutamic dehydrogenase (GLDH) has not yet been determined, but will be of the same order, because urea ^{15}N is up to 10‰ depleted if compared with serum protein, as demonstrated by the presented data.

The observed isotope disproportionation is the result of nitrogen handling in liver metabolism. A similar branching of the nitrogen pathway also occurs in other tissues but will be of lowest importance within the rapidly proliferating tissues as the small intestine or the bone marrow. Therefore, jejunum tissue protein was chosen for monitoring the mean ^{15}N abundance of the precursor amino acids.

According to the rules of isotope ratio disproportionation (4) for any set of data points (i. e., $\delta^{15}N$ of plasma protein, of jejunum protein, and of urea of any individual) the protein synthesis rate as fraction of unity for liver nitrogen metabolism was calculated. This is the quotient of ($\delta^{15}N$ of jejunum protein – $\delta^{15}N$ of urea) divided by ($\delta^{15}N$ of plasma protein $\delta^{15}N$ of urea), which is termed utilization of amino acids from the metabolic pool in

nitrogen metabolism of the liver. The course of this utilization depending on protein intake and protein source is shown in Fig. 8.

The systemic amino acids are mainly derived from protein breakdown and only to a smaller extent from the diet (10). Thus, dietary amino acids cannot be taken as the only precursor. Exogenous amino acids from the dietary protein together with the endogenous amino acids from protein breakdown form the precursor pool. Therefore, two findings from this study may be important.

i) Utilization of amino acids at zero protein intake, a condition at which only endogenous amino acids are available, is only about 70%. In the case of feeding whey protein utilization becomes much better with increasing protein intake and is maximum at requirement intake. This is observed also when casein supplemented with methionine was fed. This indicates that the pattern of amino acids available from the metabolic pool may improve depending on the amino acid pattern of the dietary protein. The effect was absent with non-supplemented casein, soy protein, and wheat gluten.

ii) Utilization at protein intake exceeding requirement resulted in nearly identical values for soy protein, casein, and wheat gluten. Utilization was about 13% higher in the case of whey protein, whereas the balance data were not different. This demonstrates, that in this case protein synthesis as well as protein breakdown is accelerated in the liver.

The data do not represent whole body protein synthesis and protein breakdown. However, by this technique the quality of amino acid pattern available from the metabolic pool is evaluated. Additionally, we assume that the transient store of amino acids in nitrogen metabolism of the liver (12) can be quantified. Protein synthesis rate in this compartment can be calculated with good approximation simply from excreted urea and from the utilization of amino acids from the metabolic pool which is determined from nitrogen isotope ratio disproportionation.

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