On the Role of Branched-Chain Amino Acids in Protein Turnover of Skeletal Muscle. Studies *in vivo* with L-Norleucine

Klaus-Joachim Schott*, Jochen Gehrmann, Ulla Pötter, and Volker Neuhoff

Max-Planck-Institut für experimentelle Medizin, Forschungsstelle Neurochemie, Hermann-Rein-Straße 3, D-3400 Göttingen, Bundesrepublik Deutschland

1. The effect of L-norleucine, an isomer of leucine, on protein metabolism *in vivo* was studied in suckling rats. Rats were injected subcutaneously with various doses of L-norleucine (0.5 and 5.0 µmol/g body wt.) every 12 h from 3 to 15 days post partum. Protein concentration, amino acid concentrations, and incorporation of [³H]tyrosine into protein were analyzed in liver, muscles of thigh and small intestine. Amino acid concentrations and insulin levels in serum were also measured.
2. At 5 days of age, norleucine induced an increase in protein concentration of skeletal muscle with an increased incorporation of [³H]tyrosine into protein indicating an accelerated protein synthesis. Changes in protein metabolism were paralleled by alterations in the amino acid pattern of this tissue.
3. When protein concentration and protein synthesis were increased in skeletal muscle, protein concentration of small intestine was decreased, accompanied by elevated levels of amino acids in tissue. Protein synthesis of small intestine was not altered by the norleucine treatment. The results suggest a close interrelationship between skeletal muscle and small intestine with respect to protein turnover.
4. The effects of norleucine were less pronounced at 10 and 15 days of age, which indicates a metabolic adaptation to the treatment.
5. Alterations in amino acid concentrations of tissue due to changes in protein metabolism were not uniform but tissue-specific.
6. Current concepts for explaining the effects of branched-chain amino acids (BCAA) on protein turnover in skeletal muscle are based on the assumption that the BCAA or leucine alone might become rate-limiting for protein synthesis in muscle under catabolic conditions. The amino acid analogue norleucine, however, cannot replace any of the BCAA in protein. Additionally, norleucine affected protein metabolism in highly anabolic organisms. Therefore, the present thoughts on this issue appear to be incomplete.

Introduction

The branched-chain amino acids (BCAA) and in particular leucine may play an important role in the control of protein turnover in skeletal and cardiac muscle. Studies *in vitro* revealed their ability of stimulating protein synthesis and inhibiting protein degradation in skeletal [1−4] and cardiac muscle [5]. Additionally, studies *in vivo* have shown that the application of BCAA led to a retention of nitrogen during starvation [6] and posttraumatic protein catabolism [7, 8]. This was thought to be due to both an increase in protein synthesis of muscle and liver and a decrease in whole body protein breakdown [9].

The mechanism, however, by which the BCAA may act upon the synthesis and degradation of protein is unresolved. Moreover, the *in vivo* effects of BCAA on protein synthesis in skeletal muscle are still controversial [10].

For the further evaluation of the role of BCAA in protein turnover of skeletal muscle *in vivo* we thought it would be helpful to investigate the effects of a potential leucine analogue on protein metabolism. L-Norleucine, the straight chain isomer of L-leucine appeared to be such a compound. It is strongly effective in inhibiting protein degradation in isolated hepatocytes thereby mimicking effects of leucine [11]. Our preliminary results then showed that norleucine, which is tolerated by animals [12, 13], induced changes in protein content and amino acid concentrations of skeletal muscle of developing rats, indicating changes in protein turnover of this tissue. Developing rats were used because protein turnover rates are highest during development

[14—17] whereby protein anabolism (growth) exceeds protein catabolism. Changes in protein turnover are likely to influence this balance between anabolic and catabolic rates and may therefore become manifest in an altered protein accumulation in tissue of developing rats. To get an overall picture of amino acid and protein metabolism, we have measured protein concentration, free amino acid concentrations and incorporation of [3H]tyrosine into protein in several tissues of the treated animals. Insulin in serum was also determined.

Materials and Methods

Adult Wistar strain rats were purchased from the Zentralinstitut für Versuchstierkunde (Hannover, FRG) and kept under standardized conditions. Rats were mated and the litters reduced to eight immediately after birth. Littermates were injected subcutaneously with various doses (0.5 μmol/g body wt. and 5.0 μmol/g body wt.) of L-norleucine (Sigma Co., St. Louis, USA) every 12 h from 3 days post partum (p.p.) until 15 days p.p. Controls received equal volumes of saline. Solutions were as follows:

A) 12.5 mM L-norleucine in 0.9% w/v NaCl, pH 7.4;
B) 0.9% w/v NaCl, pH 7.4 (control to solution A);
C) 125 mM L-norleucine in 0.5% w/v Na_2CO_3 and 0.15% w/v NaCl, pH 9.0;
D) 0.5% w/v Na_2CO_3 and 0.5% w/v NaCl, pH 9.0 (control to solution C).

The high concentration of L-norleucine (solution C) was soluble only in alkaline bicarbonate. All solutions were isotonic.

The young rats were killed by decapitation at 5, 10 and 15 days p.p. between 14:00 and 15:00 h, that is 6 h after the last injection. Skeletal muscle (muscles of thigh) and liver were removed and immediately frozen on dry ice. Two cm of the upper small intestine, from the end of duodenum, were excised, cut lengthwise and washed in ice-cold saline. The tissue was then dried on filter paper and frozen on dry ice. Trunk blood was collected, allowed to clot and centrifuged. Tissue and serum were frozen at −70 °C until analysis. Protein concentration in tissue was determined according to the method of Neuhoff *et al.* [18] which corresponds well to the Kjeldahl method. Free amino acid concentrations were determined according to the method of Neuhoff [19], in which the amino acids are dansylated and separated by two-dimensional TLC on 3×4 cm micro-polyamide

sheets followed by computer-controlled scanning fluorometry [20]. Error in measurement is within 2% and the reproducibility of the whole procedure is approximately 3—10% as regards the coefficient of variation.

Histidine is the only amino acid which cannot be quantified by this method. Methionine and arginine were ommitted from analysis because they yielded inconsistent values.

For study of incorporation of [3H]tyrosine into protein, 5 day-old rats were injected intraperitoneally with 0.5 μCi/g body wt. [3H]tyrosine 4 h after the last injection of L-norleucine (0.5 μmol/g body wt.) or saline. L-[2,3,5,6-3H] Tyrosine (specific radioactivity 84 Ci/mmol) was obtained from Amersham International (Braunschweig, FRG). Rats were kept in the dark at 34 °C and killed by decapitation 30, 60, 120 and 240 min after injection of [3H)tyrosine. Organs were removed as described above, immediately frozen on dry ice and stored at −70 °C. Tissue was homogenized 1:10 w/v in 0.4 M $HClO_4$ and centrifuged at $12,000 \times g$ and 4 °C. The supernatant was removed and frozen at −20 °C. The pellet was resuspended in 5% w/v trichloroacetic acid (TCA) and repelleted by centrifugation at $12,000 \times g$. The pellet was then resuspended in hot 5% w/v TCA and heated at 90 °C for 15 min to release label from amino-acylated tRNA. After pelleting and an additional wash in cold 5% w/v TCA, fat was extracted in chloroform/methanol (2:1, v/v) overnight. One volume of methanol was then added for pelleting of the sample. The pellet was finally washed in diethylether, dried and dissolved in 1 M-KOH. Protein content of the solution was determined according to the method of Lowry *et al.* [21]. Protein bound radioactivity was determined by liquid scintillation spectrophotometry using the rackbeta 1217 scintillation counter from LKB-Wallac (Turku, Finland) and Lumagel SB (LKB-Produkter AB, Bromma, Sweden) as scintillation cocktail. The measurement was corrected for quenching using the external channel ratio and an external standard. Incorporation of [3H]tyrosine into protein was then expressed in d.p.m./mg protein.

Free specific radioactivity of [3H]tyrosine in tissue was determined as follows. The $HClO_4$ supernatants were neutralized with 1 M-KOH and counted for radioactivity. Concentration of tyrosine was then determined by the dansylation procedure as described above. Radioactivity in supernatants was further analyzed by descending PC on Whatman 20 paper

using (1)-butanol − glacial acetic acid − H_2O (4:1:1, v/v) as solvent. Chromatograms were cut lengthwise into 1 cm pieces, the pieces eluted in 0.025 M borate (pH 8.3) overnight and the samples counted for radioactivity as described above. In muscle and small intestine, activity corresponded solely to a tyrosine standard and in liver also other activities were found. Free specific radioactivity of [³H]tyrosine in tissue was then expressed in d.p.m./nmol tyrosine.

Insulin in serum was determined by using the rat insulin radio-immunoassay kit from the NOVO Research Institute (Bagsvaerd, Denmark).

Statistical analysis of the data was performed using Student's t-test for unpaired values. In case of unequal variances the Behrens-Fisher test (d-test) was applied.

Results

Protein concentration in tissue

Protein concentration in tissues of norleucine treated and control animals is shown in Table I. Tissue-specific protein accumulation in controls was characterized by two opposing processes. In muscle, pro-tein concentration doubled between 5 and 10 days of age and remained constant until 15 days. In contrast, protein concentration of small intestine decreased about 50% between 5 and 10 days of age and rose again from 10 to 15 days. However, the 5 day-value was not reached again. In comparison, protein concentration of liver rose steadily throughout the test period.

L-Norleucine strongly affected protein accumulation in skeletal muscle and small intestine of developing rats. In both norleucine groups, protein concentration of skeletal muscle was above control values at 5 days of age whereas, at the same time, protein concentration of small intestine was below control values. Protein concentration of skeletal muscle from animals treated with the high dose of norleucine then increased steadily with age and remained slightly above control values. In animals treated with the low dose of norleucine, protein concentration of skeletal muscle fell below control values at 10 days and rose to control values at 15 days of age. By contrast, in both norleucine groups protein concentration of small intestine remained fairly constant throughout the test period.

Table I. Protein concentration in tissue of controls and experimental animals.
Groups were as follows. Controls received twice daily 0.9% w/v saline. Only the saline controls were stated since there was no difference between saline controls and bicarbonate controls (solutions B and D, see Methods section). Animals of the low dosage norleucine group received twice daily 0.5 μmol/g body wt. L-norleucine. Animals of the high dosage norleucine group received twice daily 5.0 μmol/g body wt. L-norleucine. Values are expressed as mg/g wet wt. Results are means ± S.D. from 6 animals each.
Abbreviations: *, **, ***, significantly different from controls at $P < 0.05$, < 0.01 and < 0.001, respectively.

		Muscle	Small intestine	Liver
5 day-old rats	Control	51.41 ± 4.58	130.83 ± 7.61	108.22 ± 7.20
	low dosage norleucine group	69.52 ± 4.65***	108.17 ± 5.28***	101.44 ± 4.01
	high dosage norleucine group	75.07 ± 11.55***	92.90 ± 7.53***	127.80 ± 8.28**
10 day-old rats	Control	93.60 ± 11.29	55.30 ± 11.06	127.55 ± 10.54
	low dosage norleucine group	72.70 ± 10.54**	96.07 ± 3.59***	123.28 ± 10.29
	high dosage norleucine group	106.21 ± 15.31	102.19 ± 8.03***	140.86 ± 8.68*
15 day-old rats	Control	100.18 ± 10.16	99.0 ± 3.76	146.43 ± 15.22
	low dosage norleucine group	111.48 ± 12.04	88.23 ± 6.02***	139.56 ± 16.44
	high dosage norleucine group	125.04 ± 13.81**	94.41 ± 10.29	151.53 ± 11.71

Of the tissues studied, protein concentration of liver was least affected by the norleucine treatment. Protein concentration of liver was only increased in 5 and 10 days old animals treated with the high dose of norleucine.

Incorporation of [³H]tyrosine into protein

To elucidate the effect of norleucine on protein accumulation in developing rats, as indicated by

Fig. 1. Incorporation of [³H]tyrosine into muscle protein. The upper graph shows the incorporation of [³H]tyrosine into total muscle protein of 5 day-old rats after application of label at 0 min. ●, saline controls (solution B, see Methods section), ▲, norleucine treated rats (solution A, see Methods section). Values represent means of 3 to 4 animals with bars indicating S.E.M. The lower graph shows the corresponding free specific radioactivity of [³H]tyrosine in muscle (precursor pool).

changes in protein concentration of tissue, we have studied the incorporation of [³H]tyrosine into protein of several tissues of 5 day-old treated rats. At this age the effect of norleucine on protein accumulation was most pronounced (see Table I). Tyrosine was used because it is not metabolized by skeletal muscle. Additionally, the intracellular pool of tyrosine has been shown to serve as precursor pool for protein synthesis in skeletal muscle [22].

Incorporation of [³H]tyrosine into muscle protein was highly increased in norleucine treated rats (Fig. 1) whereas in liver and small intestine no difference to controls was found (data not shown). This increase in incorporation of label may indeed reflect an accelerated protein synthesis in skeletal muscle of norleucine treated rats since the specific activity of the precursor pool was not above but slightly below control values (Fig. 1). In conclusion, the treatment with L-norleucine seems to affect specifically protein synthesis in skeletal muscle.

Free amino acid concentrations

Relative amino acid levels (% of control) in muscle, small intestine, liver, and serum of norleucine treated rats are shown in Figs. 2 to 4. The changes in amino acid concentrations of norleucine treated rats were most pronounced at 5 days of age when the protein metabolism was also most affected. In the following section, the changes in amino acid concentrations of 5 day-old treated rats are examined in detail.

Although there were some differences between the norleucine dosage groups at 5 days of age, the alterations in the amino acid pattern were rather similar (Fig. 2). In muscle, for instance, concentrations of LYS, ALA, ASP, GLU, GLN, GLY, SER, VAL and ORN were elevated in either norleucine group whereas concentrations of LEU, ILE and PHE were only increased in animals receiving the low dose of norleucine. Concentrations of ASN and THR were elevated in animals receiving the high dose of norleucine. A decrease in amino acid concentrations of skeletal muscle was only found in the group receiving the low dose of norleucine (PRO and HYP). It is interesting to note that the concentration of LEU was not altered in animals receiving the high dose of norleucine.

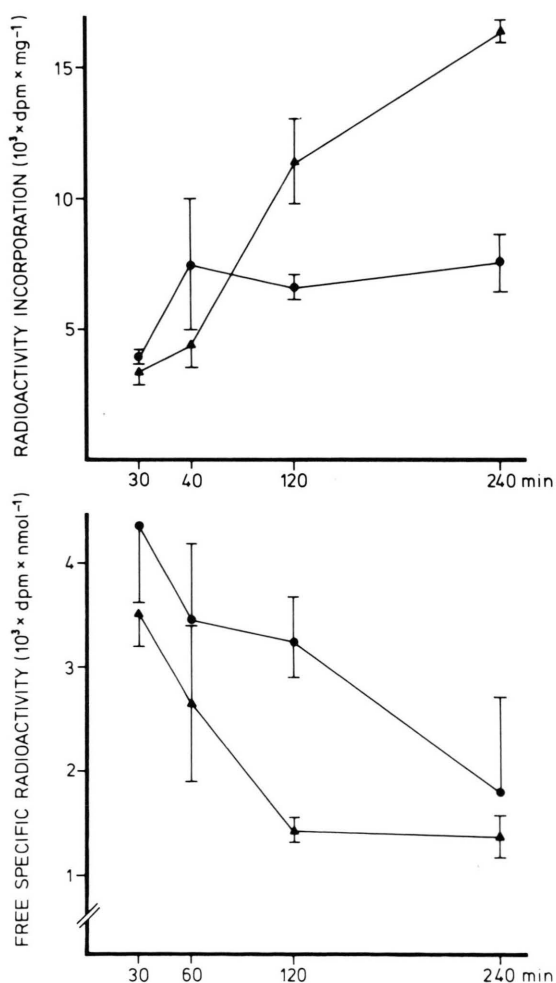The changes in the amino acid pattern of small intestine were similar to those of muscle at 5 days of
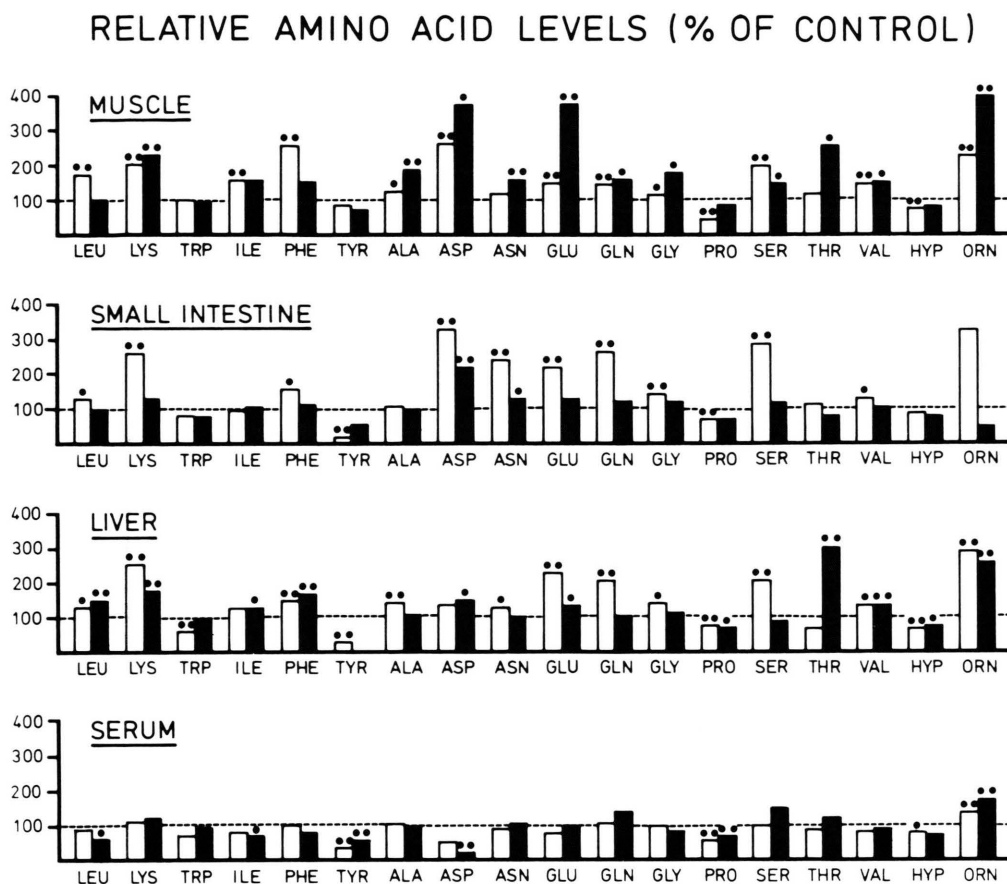
RELATIVE AMINO ACID LEVELS (% OF CONTROL)

Fig. 2. Relative amino acid levels in 5 day-old experimental animals. Open columns represent means for the low dosage norleucine group (solution A, see Methods section) and black columns represent means for the high dosage norleucine group (solution C, see Methods section). Results are expressed as % of control values and are based on 6 animals each. Abbreviations: ●, ●● Significantly different from controls at P < 0.05 and < 0.01, respectively.

age (Fig. 2). The most prominent alterations, however, were found in animals treated with the low dose of norleucine. In this group, concentrations of LEU, LYS, PHE, ASP, ASN, GLU, GLN, GLY, SER and VAL were increased whereas concentrations of TYR and PRO were decreased. In the group receiving the high dose of norleucine, only the concentrations of ASP and ASN were increased. A decrease in amino acid concentrations was not found in this group.

In liver, concentrations of LEU, LYS, PHE, GLU, VAL and ORN were increased in either norleucine group whereas concentrations of GLN and SER were only increased in the group receiving the low dose of norleucine. The concentration of THR was highly increased in animals receiving the high

dose of norleucine and concentrations of TYR and PRO were below control values in both groups.

The amino acid pattern of serum was least affected by the norleucine treatment (Fig. 2). In both norleucine groups, the concentration of ORN was increased whereas concentrations of TYR and PRO were decreased. Concentrations of LEU, ILE and ASP were below control values in the group receiving the high dose of norleucine. The changes in amino acid concentrations of tissues due to the norleucine treatment were less pronounced at later stages; however, the changes in serum remained rather constant over the test period (Figs. 3 and 4). It is interesting to note that in animals, receiving the low dose of norleucine, concentrations of several amino acids (LEU, TRP, ILE, PHE, ALA, ASN
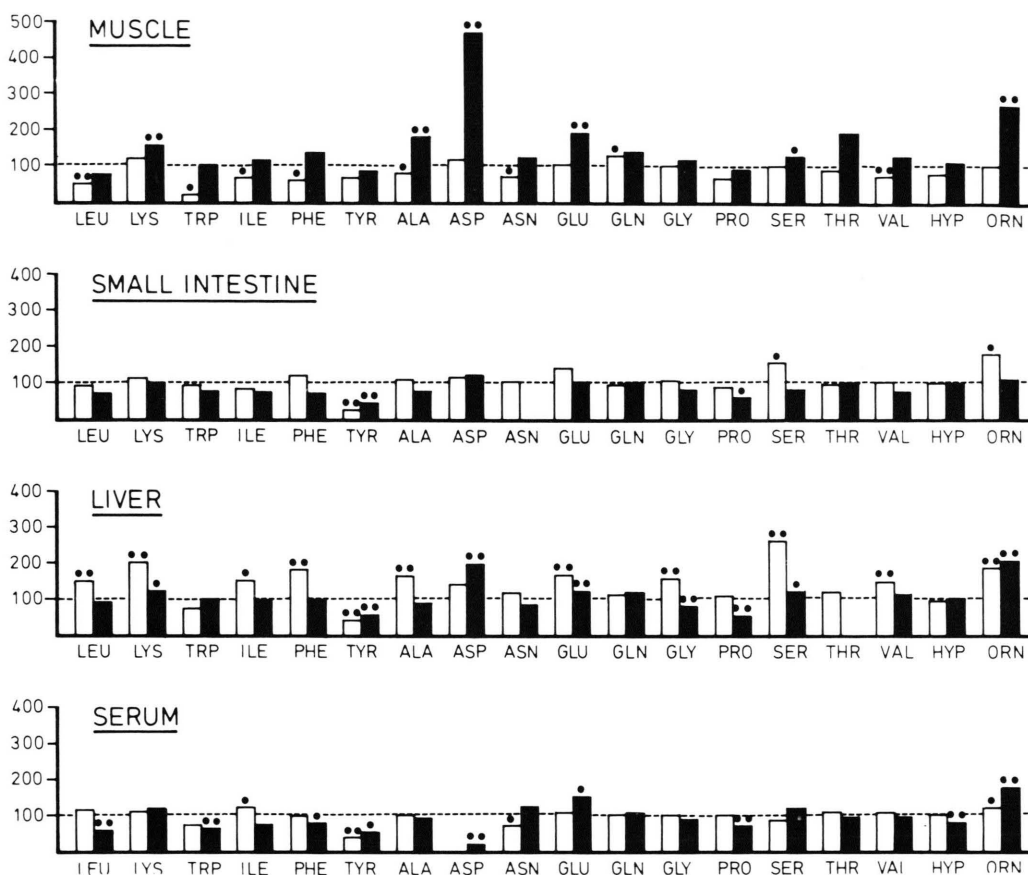
RELATIVE AMINO ACID LEVELS (% OF CONTROL)

Fig. 3. Relative amino acid levels in 10 day-old experimental animals. Open columns represent means for the low dosage norleucine group (solution A, see Methods section) and black columns represent means for the high dosage norleucine group (solution C, see Methods section). Results are expressed as % of control values and are based on 6 animals each. Abbreviations: •, •• Significantly different from controls at P < 0.05 and < 0.01, respectively.

and VAL) in skeletal muscle fell below control values at 10 days of age. In this tissue, protein concentration was also decreased when compared to controls (see above).

In summary, the alterations in protein metabolism of muscle and small intestine induced by norleucine appear to be paralleled by complex alterations in amino acid concentrations. Briefly, most amino acids in skeletal muscle were increased when protein content was also increased. In small intestine of 5 day old rats, however, amino acid concentrations were increased when protein content was decreased.

A similar relationship between amino acid concentrations and protein accumulation was also observed under physiological conditions. For instance, when protein is accumulated in skeletal muscle and

lost in small intestine from 5 to 10 days of age (see above) concentrations of LYS, ALA, ASN, GLU, GLN, GLY, SER, and ORN rose in skeletal muscle. In small intestine, concentrations of LYS, GLN and VAL rose above the 5 day-values and concentrations of TRP, PRO and ORN fell below the 5 day-values. These results are shown in Fig. 5.

Norleucine concentrations

Concentrations of L-norleucine in serum and tissue of treated rats are shown in Table II. In animals receiving the low dose, the concentration of norleucine was highest in skeletal muscle followed by the concentration in serum. In small intestine and liver of these animals only trace amounts of norleucine were detected. Concentrations of norleucine
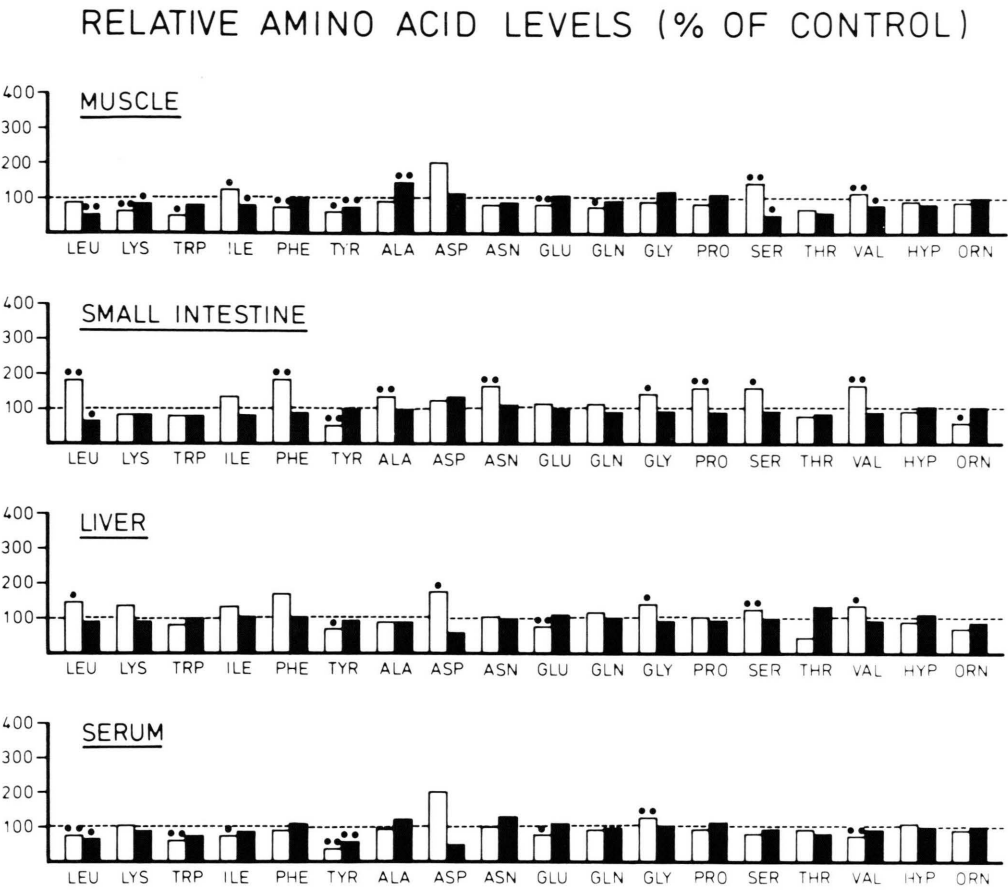
RELATIVE AMINO ACID LEVELS (% OF CONTROL)

Fig. 4. Relative amino acid levels in 15 day-old experimental animals. Open columns represent means for the low dosage norleucine group (solution A, see Methods section) and black columns represent means for the high dosage norleucine group (solution C, see Methods section). Results are expressed as % of control values and are based on 6 animals each. Abbreviations: •, •• Significantly different from controls at P < 0.05 and < 0.01, respectively.

Table II. Norleucine concentrations in experimental animals.
Groups were as follows. Animals of the low dosage norleucine group received twice daily 0.5 μmol/g body wt. L-norleucine. Animals of the high dosage norleucine group received twice daily 5.0 μmol/g body wt. L-norleucine (see Methods section). Norleucine concentrations in serum are expressed as μmol/l and concentrations in tissue are expressed as nmol/g wet wt. Results are means ± S.D. from 6 animals each.
Abbreviations: T.A., Trace amount.

		5 Day-old rats	10 Day-old rats	15 Day-old rats
low dosage	serum	53 ± 16	32 ± 9	18 ± 3
norleucine group	muscle	166 ± 18	73 ± 39	166 ± 9
	small intestine	T.A.	T.A.	0
	liver	T.A.	T.A.	0
high dosage	serum	347 ± 162	337 ± 127	369 ± 76
norleucine group	muscle	365 ± 153	596 ± 159	287 ± 71
	small intestine	221 ± 83	172 ± 76	176 ± 43
	liver	308 ± 95	322 ± 110	235 ± 111

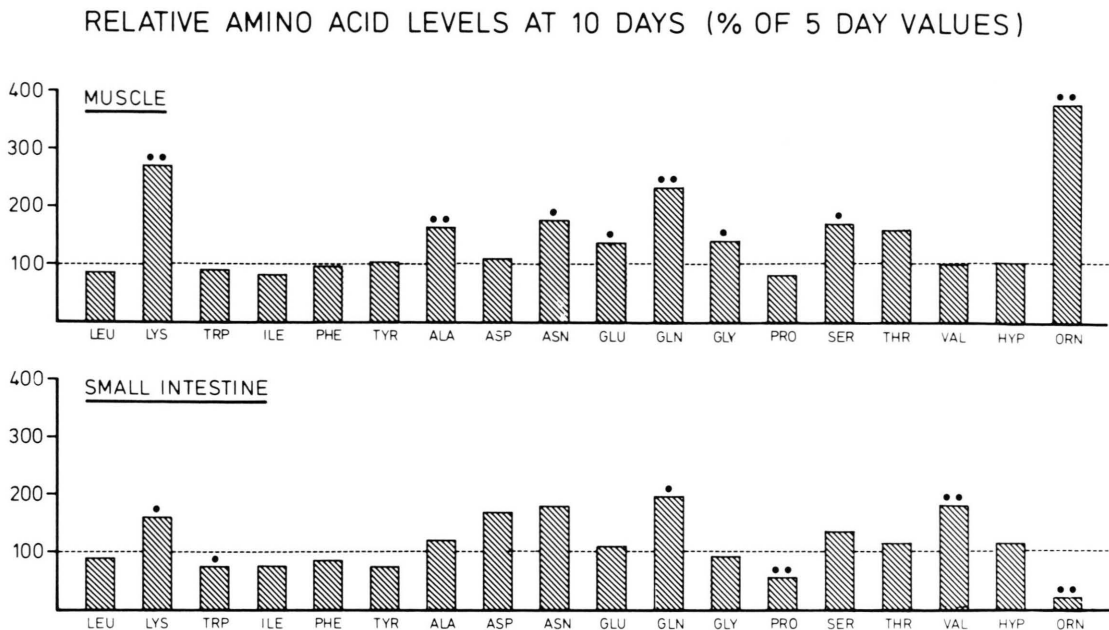## RELATIVE AMINO ACID LEVELS AT 10 DAYS (% OF 5 DAY VALUES)

Fig. 5. Alterations in amino acid pattern of controls with age. Columns represent relative amino acid levels of 10 day-old controls* expressed as % of the 5 day-values. Values were based on 6 animals each. Abbreviations: •, •• Significantly different from 5 day-values at P < 0.05 and < 0.01, respectively.
* (Solution B, see Methods section).

in serum declined with age whereas concentrations in muscle fell about 50% at 10 days of age and raised again to the 5 day-value at 15 days of age.

In animals receiving the high dose, norleucine was found at similar concentrations in serum and all tissues investigated. In these animals, concentrations of norleucine were generally higher than in those animals treated with the low dose of norleucine. Concentrations of norleucine remained constant throughout the test period in this group, except for

the concentration of norleucine in muscle which showed a peak at 10 days of age.

Of the tissue investigated, skeletal muscle seems to accumulate norleucine at the highest rate. This is in agreement with studies of Hassan and Greenberg [23].

Insulin in serum

The concentration of insulin in serum of controls and norleucine treated animals is shown in Table III.

		5 Day-old rats	10 Day-old rats	15 Day-old rats
saline	low dosage norleucine group (solution A)	0.88 ± 0.52 (5)	0.69 ± 0.32 (5)	0.53 ± 0.11 (5)
	control (solution B)	0.98 ± 0.64 (6)	0.73 ± 0.39 (5)	0.81 ± 0.45 (5)
	high dosage norleucine group (solution C)	0.69 ± 0.21 (6)	0.43 ± 0.12 (3)	0.85 ± 0.12 (4)
bicarbonate	control (solution D)	0.60 ± 0.18 (5)	0.36 ± 0.18 (4)	1.06 ± 0.47 (5)

Table III. Insulin concentrations in serum of controls and experimental animals.
Groups were characterized by injection solutions (see Methods section). Values are expressed as ng/ml and represent means ± S.D. with number of animals in parentheses.

The concentration of insulin in serum of saline controls (solution B) was constant throughout the test period. In contrast, concentrations of insulin in serum of bicarbonate controls (solution D) were different from the values of saline controls (solution B) and varied throughout the test period. However, there was no difference between controls and norleucine treated animals.

Discussion

Investigating the effects of a possible leucine agonistic substance on protein turnover promised to give a new insight into the mechanisms by which the BCAA may regulate protein metabolism in skeletal muscle since the present concepts for explaining these effects are solely based on the specific metabolic fate of BCAA in this tissue [1, 24, 25]. The BCAA are chiefly metabolized in skeletal muscle and contribute to the energy metabolism in this tissue to a large extent, particularly when there is a need for oxidizable substrates [24]. It has been concluded that, under catabolic conditions, the BCAA might become rate limiting for protein synthesis. Hence, Tischler *et al.* [26] studied the possible regulatory role of leucyl-tRNA in protein turnover of skeletal muscle. The ratio of charged to uncharged tRNA, in this context, has been shown to correlate with rates of protein synthesis [27, 28] and protein degradation [29] in mammalian cells.

However, a role of leucyl-tRNA in protein turnover of skeletal muscle could not be confirmed [26]. Others have pointed to the ability of BCAA or leucine alone to increase the ratio of polysomes to subunits and monomers in skeletal muscle from fasted rats [25]. From this it was concluded that the BCAA may regulate protein synthesis at the translational level. This might agree with earlier findings of Munro [30], who could show that ribosomal aggregation in liver is dependent on an appropriate amino acid supply. He proposed a concept ascribing a regulatory role to that amino acid which is least available for protein synthesis, that is, tryptophan in liver.

However, our results may query the present thoughts on this issue because norleucine, which mimics effects of leucine on protein turnover, is an unnatural free amino acid for animals being not able to replace leucine in protein. Hitherto, norleucine has only been shown to be edited by the [Met]tRNAs in

E. coli [31, 32]. Therefore, it seems unlikely that the effects of norleucine on protein turnover are mediated through the charging of tRNA. Furthermore, norleucine cannot be a rate-limiting amino acid for protein synthesis. Norleucine, however, is not toxic to animals and is readily metabolized in the rat [23, 33] to yield glucose [12, 13]. It is transported into cells via the A and L system for amino acid transport [34].

According to our results, L-norleucine undoubtedly affected protein turnover in skeletal muscle of developing rats. These effects were most pronounced at 5 days of age and leveled off a later stages which is probably due to a metabolic adaptation. We have therefore focused this investigation on 5 day-old rats. L-Norleucine induced an increase in protein synthesis of skeletal muscle of 5 day-old rats, in which protein content of muscle was also increased. Furthermore, protein degradation in isolated hemidiaphragms of 5 day-old, norleucine treated rats was lower than in controls (unpublished results; these experiments were done according to Fulks *et al.*, ref. 2).

Preliminary results from gel electrophoresis of muscle protein indicate a general shift in protein synthesis of skeletal muscle since no gross differences to controls were found (unpublished results).

However, the question arises if the effects of norleucine could be caused by an increase in concentrations of leucine in tissue rather than by the substance itself. Although norleucine is a poor substrate of BCAA-transaminase [35, 36] it might interfere with the catabolism of leucine thereby increasing its concentration in tissue. However, there was no correlation between concentrations of leucine and protein metabolism in muscle. For instance, in 5 day-old animals receiving the low dose of norleucine the concentration of leucine in muscle was increased about 75%. By contrast, in 5 day-old animals treated with the high dose of norleucine the concentration of leucine was not altered (see Fig. 2). In both groups, however, protein concentration of muscle was increased when compared to controls (see Table I). In diaphragm of norleucine treated animals, in which protein degradation was decreased, the concentration of leucine was in the control range (unpublished results). Therefore, it seems reasonable to assume that norleucine or a metabolite is the effective compound.

It is noteworthy in this context that several authors [10, 37] have found no correlation between concen-

trations of BCAA in plasma and tissue and rates of protein synthesis *in vivo*.

We have then investigated the relationship between protein turnover and amino acid concentrations of tissue in general. Thus, the changes in protein metabolism of skeletal muscle of 5 day-old norleucine treated rats paralleled the changes seen in amino acid concentrations of this tissue. Briefly, most of the amino acids were increased when protein concentration and protein synthesis were also increased. This might agree with findings of Miller [38], who found coinciding peaks of concentrations of free amino acids and protein synthesis in liver and brain of developing rats. The rise in amino acid concentrations was thought to be due to an increased uptake of amino acids into tissue for maintaining protein synthesis. According to our results, growth and protein accumulation in skeletal muscle of suckling rats appear to be paralleled by increasing concentrations of LYS, ALA, ASN, GLU, GLN, GLY, SER, and ORN (see Fig. 5). In skeletal muscle, however, increased concentrations of ALA, GLU, GLN, ASP, ASN, ILE and VAL are likely to signal a rapidly turning over of amino nitrogen [39, 40]. The meaning of these results remains to be clarified.

The findings in skeletal muscle contrast with the findings in small intestine. For instance, in small intestine of 5 day-old animals treated with the low dose of norleucine, concentrations of several amino acids (LEU, LYS, PHE, ASP, ASN, GLU, GLN, SER, and VAL) were increased (Fig. 2); however, protein concentration was decreased and protein synthesis was not different from controls. In these animals, small intestine seems to be in a more catabolic state of protein metabolism. Increased concentrations of amino acids may then indicate an increased protein degradation in small intestine. A similar relationship between protein accumulation and amino acid concentrations of small intestine was also observed in controls from 5 to 10 days of age (see Table I and Fig. 5). However, it might be possible that amino acid concentrations in small intestine also reflect amino acid uptake from the intestinal juice. Since skeletal muscle accounts for about 50% of whole body protein turnover [41] and amino nitrogen [42] an acceleration of protein synthesis in skeletal muscle with a rise in protein concentration will drastically increase the need for amino acids in the whole organism. For instance, in the suckling period when growth rates are highest [38] the pool of amino acids

in the lumen of small intestine declines [43]. However, amino acid concentrations in tissue of small intestine increase during this period indicating an exhaustion of dietary amino acids under conditions of rapid growth [43]. Accordingly, the uptake of amino acids by small intestine is high in developing rats [44]. These results suggest a close interrelationship of skeletal muscle and small intestine during development. Under conditions of rapid growth, the need for amino acids in skeletal muscle appears to result in an increased mobilization of amino nitrogen in small intestine involving both protein degradation and amino acid uptake.

An increased supply of amino acids from the intestinal tract may also be the cause of elevated amino acid levels in liver of treated animals which were concordant with those in small intestine. However, the high dosage of norleucine induced an increase in protein concentration of liver at 5 and 10 days of age. The alterations in amino acid concentrations of liver could, therefore, be due to an altered protein metabolism of this tissue as well. In this context, norleucine has been shown to inhibit protein degradation in isolated hepatocytes thereby mimicking effects of leucine [11].

In summary, changes in amino acid concentrations of tissue do not predict the state of protein turnover, *i.e.* anabolism vs catabolism, in a certain tissue. Nevertheless, amino acid concentrations in tissue appear to respond to changes in protein turnover in a complex and tissue-specific manner.

Alterations in amino acid transport due to norleucine are unlikely to play a role in these experiments because changes in amino acid levels of tissue were also seen when concentrations of norleucine in serum and tissue were low or near to zero. To rule out the possibility that norleucine might act on amino acid and protein metabolism by stimulating the release of insulin, we have finally measured insulin in serum of controls and norleucine treated animals. However, insulin levels in serum of norleucine treated rats were in the control range.

Concluding Remarks

A regulatory role of amino acids in protein turnover of skeletal muscle has hitherto been reported for BCAA or leucine alone. In this respect, norleucine which is chemically related to leucine appears to be a functional analogue of leucine. However, the

effects of norleucine on protein turnover in skeletal muscle cannot be interpreted on the basis of current concepts for explaining the effects of leucine on protein turnover.

These rest upon the assumption that leucine might become rate limiting for protein synthesis under catabolic conditions. Norleucine, however, cannot replace any of the BCAA in protein. Furthermore, norleucine, induced changes in protein turnover of skeletal muscle in a highly anabolic, growing organism. Thus, it seems reasonable to assume that the current concepts for explaining the effects of BCAA on protein turnover in muscle are somehow incomplete. Further studies with L-norleucine might elucidate the molecular mechanism by which amino acids can influence protein turnover in tissue.

Acknowledgements

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