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Norleucine: A Branched-Chain Amino Acid Analog Affecting Feeding Behavior of Rats

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TEWS, J. K., J. J. REPA AND A. E. HARPER. *Norleucine: A branched-chain amino acid analog affecting feeding behavior of rats.* PHARMACOL BIOCHEM BEHAV **35**(4) 911–921, 1990.—Norleucine, an isomer of leucine and isoleucine and a potent competitor of large neutral amino acid transport into brain, thereby depleting certain amino acid pools, was tested for its effects on growth and feeding behavior of rats fed an amino acid diet limiting in leucine. Growth and food intake were depressed in proportion to the dietary level of norleucine (0.2 to 1.1% of the diet). With suboptimal amounts of indispensable amino acids, leucine at 150% of the requirement reversed the effects of 0.2 and 0.5% norleucine; slight excesses of the other indispensable amino acids were required with extra leucine for maximum growth with 1.1% norleucine. Rats almost exclusively preferred the control to the norleucine diet, but not if the latter diet also contained leucine. Rats also strongly selected a nonprotein rather than norleucine diet when this was the first available choice. If the first choice was between the nonprotein and control diets, rats later almost exclusively selected the norleucine-containing rather than the nonprotein diet for varying periods (2 to 6 days). These studies suggest that amino acid analogs may be useful agents in the study of animal behavior associated with changes in brain amino acid pools.

Amino acid analog Behavior Diet Food choice Food intake Growth Large neutral amino acids Leucine Norleucine

ADDITION to a low protein diet of a mixture of analogs of the large neutral amino acids (LNAA) reduces food intake and growth of young rats; these effects are minimized by raising the protein content of the diet (23). Homoarginine alone, added to a lysine-limiting amino acid diet, is also associated with reduced food intake and growth; additional dietary lysine improves growth and efficiency of food utilization (25).

Analogs, although they may be metabolized by the rat (4, 5, 18, 29), are not generally utilized for protein synthesis. When they are present in the diet their concentrations are high in plasma, and selective decreases occur in some tissue amino acid concentrations. Thus, norleucine can selectively reduce concentrations of the LNAA in rat tissues, especially the branched-chain amino acids (BCAA) and especially in brain (23,24). Similarly, the cationic amino acid homoarginine can lower concentrations of the natural basic amino acids without a corresponding effect on the neutral amino acids (24,25). The homoarginine-associated, selective reductions in levels of each basic amino acid in brain and in the ratios of the brain concentrations to those in plasma fits with the concept that the effects of feeding diets containing disproportionate amounts of amino acids include competition for amino acid entry into the brain from the blood. The validity of this concept was established by experiments showing that the rate of entry of radioactive lysine or valine from the blood into the brain of rats was reduced by diet-induced elevations of plasma concentrations of the analogs homoarginine or norleucine, respectively, when rats received a diet correspondingly limited in lysine or valine (21). These transport effects are specific as influx of the cationic lysine was not reduced by adding small neutral serine or large neutral tyrosine to the diet, nor was valine influx depressed by dietary serine or cationic ornithine.

Quantitative aspects of the responses of rats to the various dietary disproportions of amino acids which have been used to alter growth and feeding behavior have not been well studied. In order to examine further the effects of different dietary levels of amino acids on these variables we have tested amino acid diets limiting in leucine and containing various proportions of norleucine, a leucine and isoleucine isomer and an effective competitor for transport of LNAA across the blood-brain barrier (21,22). Diets containing graded amounts of leucine have also been tested for their ability to reverse the adverse effects of norleucine. We have also examined diet selections by rats offered choices between various diets supplemented with leucine or norleucine.

Our results show clear effects of different dietary levels of

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norleucine or leucine on feeding behavior and growth, and also demonstrate either avoidance or acceptance of norleucine-containing diets depending on diet composition and on the available diet choices. These studies suggest that amino acid analogs such as norleucine can be useful tools in investigations of physiological responses to dietary amino acids that alter brain amino acid pools.

METHOD

Studies on growth and 24-hr food intakes were performed with young male rats of the Sprague-Dawley strain (King Animal Laboratories, Oregon, WI or Harlan Sprague-Dawley, Madison, WI). They were housed individually in wire mesh cages in a temperature-controlled room lighted from 0700 to 1900 hr, with food and water available at all times. The rats were first adapted to either control diet A or B as described in Table 1. After a 3-day adaptation period the rats were separated into 8 groups of 6 rats each and were fed appropriate amino acid-supplemented diets (Table 1; 4 separate experiments). At this time animal weights ranged from about 70 to 80 g. Total food intakes and body weights were measured each day for 10 days.

In the first experiment rats were adapted to the amino acid control diet A in which leucine, as the most limiting amino acid, was present at 65% of the requirement for growth of rats, while other indispensable amino acids were included at 75% of the requirements. The rats were then divided into groups and fed the control diet or this diet containing different levels of norleucine, with or without additional leucine at 150% of the requirement (Experiment 1, supplements 1–7, Table 1).

In the remaining 3 experiments rats were adapted to the amino acid control diet B in which leucine was again present at 65% of the requirement, while the other indispensable amino acids were added at 125% of the requirements. In the second experiment rats were then fed control diet B, again with different levels of norleucine and added leucine (Experiment 2, supplements 1–7, Table 1). In the third experiment increasing amounts of dietary leucine were added to diets containing a constant amount of norleucine (Experiment 3, supplements 1 and 6–11, Table 1). In the final experiment diets containing norvaline in amounts equimolar to norleucine, also with or without added leucine, were tested for their effects on growth and food intake (Experiment 4, supplements 1, 12–17, Table 1).

In other studies feeding behavior was observed for separate sets of 5 male rats each. The animals were acclimated to a computerized feeding system for several days before allowing them a choice, usually for 6 days, between various pairs of diets in sequence; diets were offered each day starting at the end of the light period. In an attempt to lessen possible preference for a familiar diet, each diet choice was usually preceded by providing for at least 2 days the appropriate control diet flavored with anise extract [0.5% of diet; (10)] in both food cups. Times of eating and the amount of diets selected were monitored every minute by an Apple IIe computer interfaced with 10 Mettler balances (model P2000; two balances per rat) as described elsewhere (28). Rats ate a total of less than 0.1 g diet if no intake was recorded. The recorded data for each 24-hr period were analyzed by a computer program which produced bar graphs showing total food consumed by individual rats from each of 2 diets during succeeding 30-min intervals. Another program tabulated daily feeding patterns during the dark and light periods (average number, size and duration of meals, intermeal interval and rate of food consumption for each diet). Choices included those between pairs of diets selected from Table 1, or between one of those diets and one containing no protein or amino acids (other ingredients as described in Table 1).

Amino acid analyses were performed on sulfosalicylic acid extracts of plasmas and brains (cerebellums omitted) by ion exchange chromatography (Beckman Model 119CL). Tryptophan concentrations were determined by a fluorometric procedure (3,6).

Analysis of variance (ANOVA) with corrections for repeated measures was performed on the growth and food intake data of Experiments 1–4; multivariate (MANOVA) tests for time and its interactions were based on Wilks' lambda statistic (19). Fisher's protected LSD test was used to determine differences among the 10-day totals ($p<0.05$). Differences between daily values for the various feeding indices were determined by the paired t-test (dark period only). Differences among amino acid concentrations were determined by ANOVA followed by the LSD test.

TABLE 1

AMINO ACID SUPPLEMENTS TO CONTROL DIETS

Amino Acid Added	Supplement Number (g/100 g diet)						
	Experiment 1, Control Diet A						
	1	2	3	4	5	6	7
Leucine	0.64		0.64		0.64		0.64
Norleucine		0.2	0.2	0.5	0.5	1.12	1.12
	Experiment 2, Control Diet B						
	1	2	3	4	5	6	7
Leucine	0.64		0.64		0.64		0.64
Norleucine		0.2	0.2	0.5	0.5	1.12	1.12
	Experiment 3, Control Diet B						
	6	8	9	10	11	1	7
Leucine		0.26	0.26	0.45	0.45	0.64	0.64
Norleucine	1.12		1.12		1.12		1.12
	Experiment 4, Control Diet B						
	1	12	13	14	15	16	17
Leucine	0.64		0.64		0.64		0.64
Norvaline		0.18	0.18	0.45	0.45	1.0	1.0

Diets contained (g per 100 g diet): vitamin mixture, 0.5 (17); corn oil, 5; mineral mixture, 5 (17); choline chloride, 0.2; amino acids as indicated below; and equal amounts of glucose monohydrate and cornstarch to make 100 g.

Control diet A: 8% L-amino acid mixture with leucine at 65% and other indispensable amino acids at 75%, respectively, of requirements for growth (1), with the remainder from dispensable amino acids; amino acid (g) per 100 g diet A: arginine·HCl, 0.45; histidine, 0.225; isoleucine, 0.375; leucine, 0.488; lysine·HCl, 0.525; methionine, 0.45; phenylalanine, 0.60; threonine, 0.375; tryptophan, 0.113; valine, 0.45; glutamate, 1.62; glycine, 1.08; asparagine, 0.278; and aspartate, alanine, serine, tyrosine, proline and cystine at 0.162 each.

Control diet B: 8% L-amino acid mixture with leucine at 65% and other indispensable amino acids at 125%, respectively, of requirements for growth (1), with the remainder from dispensable amino acids; amino acid (g) per 100 g diet B: arginine·HCl, 0.75; histidine, 0.375; isoleucine, 0.625; leucine, 0.488; lysine·HCl, 0.875; methionine, 0.75; phenylalanine, 1.0; threonine, 0.625; tryptophan, 0.188; valine, 0.75; glutamate, 0.646; glycine, 0.43; asparagine, 0.111; and aspartate, alanine, serine, tyrosine, proline and cystine at 0.065 each.

Each diet also contained sodium acetate in amounts equimolar to the amino acid hydrochlorides. L-Norleucine and L-norvaline were used in equimolar amounts and were added at the expense of the carbohydrates.

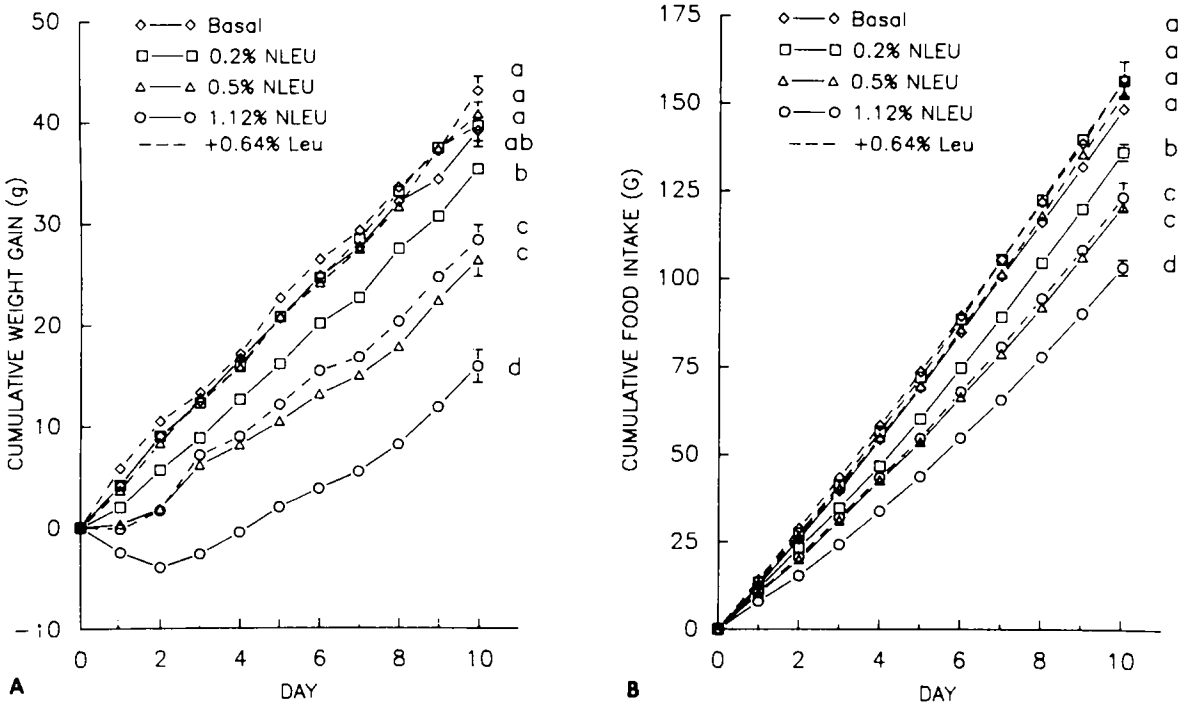

FIG. 1. Cumulative effects of different levels of dietary norleucine with or without added leucine on body weight (A) and food intake (B) of rats fed for 10 days the diets described in Table 1, Experiment 1; the control (basal) diet contained leucine at 65% of the requirement and other indispensable amino acids at 75% of the requirements (control diet A). Different letters on right of graphs indicate significant differences in the 10-day total values ($p<0.05$).

RESULTS

Growth and Food Intake

Growth of rats in Experiment 1 was increasingly depressed as dietary content of norleucine was raised from 0 to 1.1% of the diet (Fig. 1A). Total weight gain for rats fed 1.1% norleucine was reduced to 40% of that observed for the control rats. Additional dietary leucine (at 150% of the requirement) did not significantly increase weight gain of the control rats, but completely prevented the adverse effects of all but the highest level of norleucine; total weight gain of rats fed 1.1% norleucine plus leucine was about 65% of that of the leucine-supplemented control rats. Responses to leucine, $F(9,32)=4.44$, $p<0.008$, and norleucine, $F(27,94)=3.47$, $p<0.0001$, both differed with time over the 10-day period. Although there was strong evidence for interaction among these amino acids, $F(3,40)=6.73$, $p<0.001$, there was no evidence that the interaction varied with time.

Food intakes in the first experiment were also increasingly depressed as norleucine content of the diet was raised (Fig. 1B), but only the response to norleucine differed with time, $F(27,94)=1.91$, $p<0.012$. Again, except for rats fed 1.1% norleucine, total food intakes of rats fed additional leucine did not differ from control values.

Because of the previously observed striking depressions in tissue BCAA concentrations in rats fed norleucine (22,24), Experiment 1 was partially repeated and extended by feeding increased amounts of valine and isoleucine (150% of requirement) and/or leucine (250% of requirement) with or without norleucine at 1.1% in the control diet used in Experiment 1, Table 1 (12 groups of rats). The marked reduction in weight gain caused by norleucine was not alleviated by raising the valine and isoleucine content of the diet. The further increase in dietary leucine to 250% of the requirement also did not lessen the effects of norleucine beyond the improvement seen in Experiment 1 (Fig. 1) after feeding leucine at 150% of the requirement (results not shown). This experiment therefore suggested that the adverse effects of norleucine in Experiment 1 were unlikely to be related only to inadequate dietary intake of the BCAA.

When dietary content of all indispensable amino acids except leucine was raised in Experiment 2 (Table 1), norleucine again caused dose-related depressions in growth and food intake (Fig. 2A, B). Thus, rats fed the diet containing 1.1% norleucine gained about 25% as much weight as the control rats during the 10-day study. When extra dietary leucine was added, total weight gain of the control rats increased and the growth depression caused by each of the tested levels of norleucine was completely prevented. Total food intakes of rats receiving norleucine were significantly depressed (to 64% of control in rats fed 1.1% norleucine); addition of leucine to these diets completely prevented these depressions. For both these variables only the effects of leucine differed with time [$F(9,32)=2.53$, $p<0.026$ and $F(9,32)=2.83$, $p<0.014$ for effects on weight and food intake, respectively].

In order to determine how effective were different levels of dietary leucine in reversing the effects of norleucine, the rats of Experiment 3 were fed diet B containing 4 levels of leucine in the presence or absence of 1.1% norleucine (Fig. 3). Weight gains were increased by similar amounts over the control value when rats were fed the control diet containing leucine at 100, 125 or 150% of the requirement. As in the second experiment, total weight gain in the presence of norleucine was reduced to 25% of the control value when the rats received control diet B which contained leucine at 65% of the requirement. Norleucine added to the diet containing leucine at 100% of the requirement did not significantly reduce weight gain; however, rats fed diets contain-

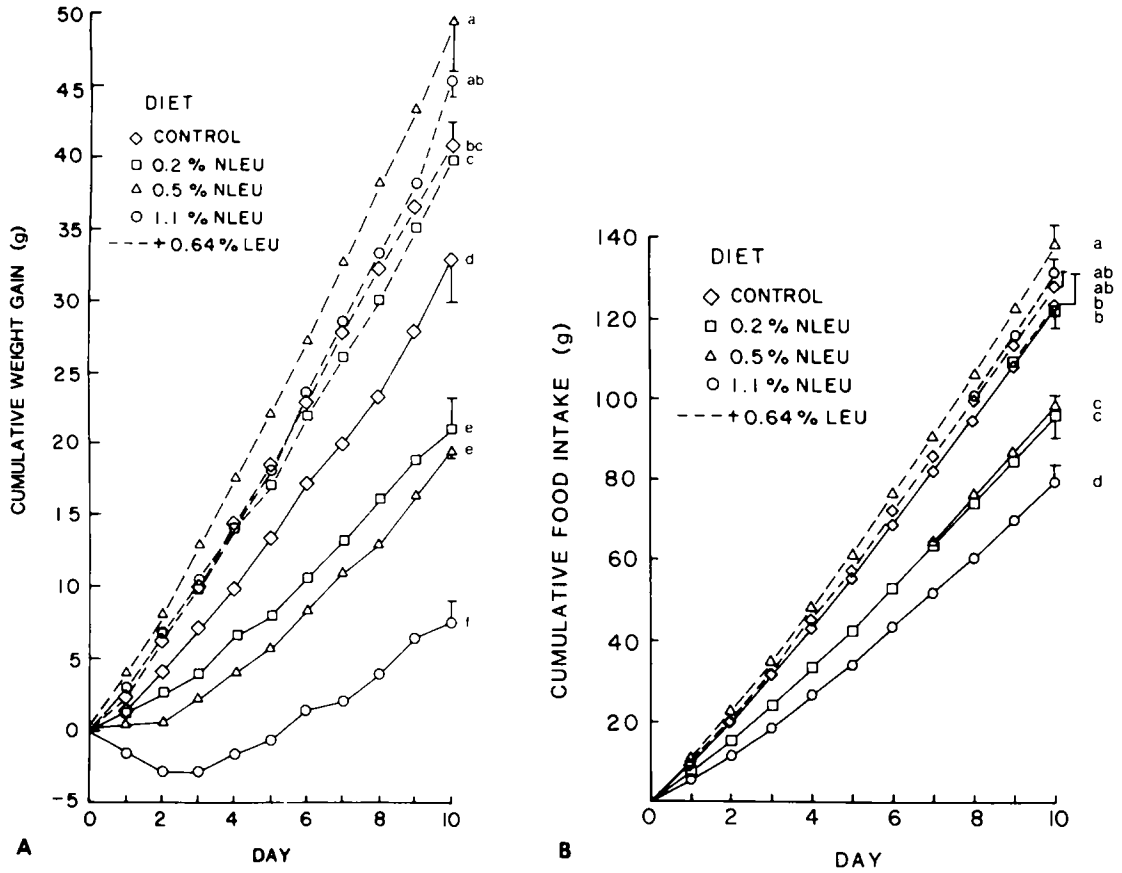

FIG. 2. Cumulative effects of different levels of dietary norleucine with or without added leucine on body weight (A) and food intake (B) of rats fed for 10 days the diets described in Table 1, Experiment 2; the control diet contained leucine at 65% of the requirement and other indispensable amino acids at 125% of the requirements (control diet B). Different letters on right of graphs indicate significant differences in the 10-day total values ($p<0.05$).

ing norleucine plus leucine at either 125 or 150% of its requirement gained more weight than those fed norleucine plus leucine at 100% of its requirement. Thus, dietary leucine in amounts corresponding to those of the remaining indispensable amino acids (125% of requirements) was probably required for complete prevention of the deleterious effects of norleucine. In this experiment the responses to norleucine or leucine each differed with time, $F(9,32)=5.27$, $p<0.0002$, and $F(27,94)=2.22$, $p<0.0025$, respectively. The interaction between these amino acids also varied over time, $F(27,94)=2.01$, $p<0.007$.

Food intakes of control rats in Experiment 3 were increased by raising dietary leucine content above 65% of the requirement. Norleucine clearly reduced food intake when rats received leucine at 65% of the requirement, but this effect was eliminated when dietary leucine was also included at 125 or 150% of the requirement (Fig. 3B). Only the response to leucine differed with time, $F(27,94)=2.00$, $p<0.008$.

Norvaline, a norleucine homolog and a valine analog, was also tested for its ability to depress growth and food intake of rats adapted to amino acid control diet B (Experiment 4, Table 1; identical in design with Experiment 2). This experiment demonstrated that norleucine was more potent than norvaline as a leucine antagonist, as only the highest dietary level of norvaline (1% of the diet) significantly reduced total growth (to 64% of control). Total food intake of this group was reduced to 75% of the control value. Both these effects were completely prevented when dietary leucine was raised to 150% of the requirement (results not shown).

Feeding Behavior

After receiving the anise-flavored 8% amino acid control diet B in both food cups for 3 days rats were allowed to choose between the unflavored control diet B with or without added norleucine (1.1%, supplement 6, Table 1). On the first day of this choice (Fig. 4) 4 of the 5 rats clearly ate more of the control diet than of the diet containing norleucine. After the first 12-hr dark period the rats almost completely avoided the latter diet, whereas considerable intake of the control diet occurred during the last half of the first day (light period). During the following 5 days some rats occasionally completely avoided the norleucine-containing diet (not shown); by days 5 and 6 they consumed about 7- to 10-fold as much of the control diet as of the one containing norleucine.

During the dark period of the first day of this choice there were no clear differences in the measured indices of feeding behavior (Table 2A). However, combined intake from the unflavored control and norleucine diets during the dark period of the first day was about half that from the anise-flavored control diet (in both cups) on the preceding day (not shown). On day 2 of the choice the rats ate from the control diet during the dark period an average of at least 6 times as much food in 5 times as many meals as from the norleucine diet; these meals lasted about 3 times as long as those from the norleucine diet. Most rats completely avoided the

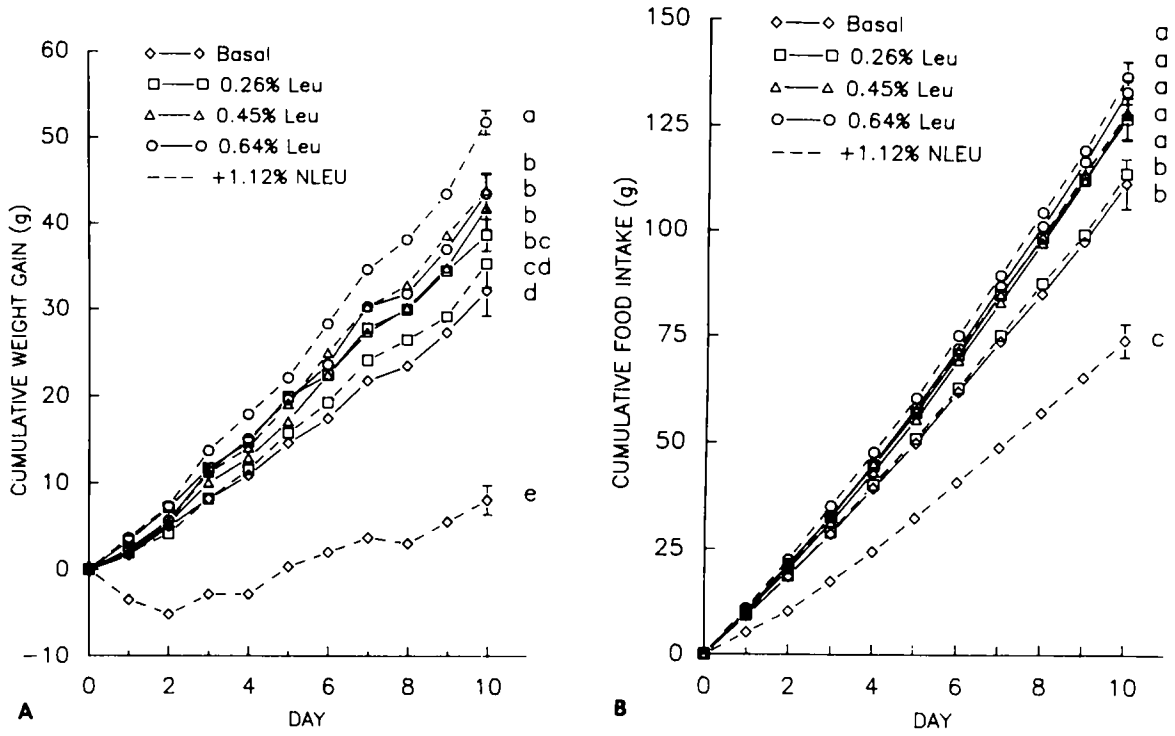

FIG. 3. Cumulative effects of different levels of dietary leucine with or without added norleucine (1.1% of diet) on body weight (A) and food intake (B) of rats fed for 10 days the diets described in Table 1, Experiment 3. The control diet was that described in Fig. 2. Different letters on right of graphs indicate significant differences in the 10-day total values ($p<0.05$).

norleucine diet during the light period.

These rats were also offered a series of further choices; results are shown for a single rat for the first day of each new choice (Fig. 5). The top row shows that this rat chose primarily during the dark period approximately equal amounts from each food cup containing the anise-flavored control diet B (i.e., there was no position preference). On the following day (row 2) the rat ate only 0.2 g of the norleucine diet (not avoided on the first day of exposure to this diet; Fig. 4, row 5) before consistently selecting the diet containing the same amount of norleucine plus additional leucine (supplement 7, Table 1). A similar preference was obtained for all 5 rats for each of 4 days. When the choice was between the norleucine + leucine and the leucine-supplemented diets 2 rats, including the one shown in Fig. 5, consistently ate during a 6-day period little or none of the previously selected norleucine + leucine diet and instead primarily chose the leucine-containing diet as on day 1 (row 3). However, 3 of the 5 rats generally ate substantial amounts of the former diet throughout the 6-day test period. When the choice was between the leucine-limiting control diet and one containing both norleucine and leucine (row 4) marked preferences often did not occur. During the last 4 days of the 6-day choice 3 of the 5 rats clearly preferred the norleucine + leucine diet, while 2 of the rats made the opposite selection; both of these diets were usually sampled. Finally, the rat described in Fig. 5 did not clearly select differently between the control diet or this diet containing added leucine on day 1 (row 5); on the following 5 days 3 or 4 of the 5 rats generally selected either more of the leucine-containing than the control diet or ate similar amounts from each diet.

These choice patterns were much less distinctive or even absent when norleucine was added at only 0.2% of the diet (supplement 2, Table 1), a reasonable response in view of the relatively small effect of this dose on growth and food intake (Figs. 1 and 2).

Early studies on amino acid imbalance, in which a mixture of all but one of the indispensable amino acids was used to induce the imbalance, showed that rats usually prefer to eat a nonprotein diet rather than a given imbalanced diet (8, 13, 14). Because of the aversive qualities of the norleucine-containing, leucine-limiting diet (Figs. 4 and 5), we also carried out with different rats a new series of choice studies in which one of the available diets was protein-free.

The single rat shown in the top row of Fig. 6 ate only 0.4 g of the 8% amino acid diet containing norleucine while consuming 6.2 g of the nonprotein diet; this choice was made within the first few hours of the first feeding period after prior exposure to the anise-flavored control diet B. During the first dark period the 5 rats chose an average of 9-fold as much of the nonprotein diet as of the amino acid diet containing norleucine; the rats ate at least 4 times as many meals which tended to be larger and last longer (Table 2B). By the second day, differences in these indices often were more striking than on day 1. Thus, the rats consumed 30-fold as much of the nonprotein diet in longer and larger meals occurring 5 to 6 times more frequently than those from the norleucine diet. The rates of eating (g diet/min) did not differ.

After an intervening exposure to the anise-flavored control diet the rats were given a choice between the nonprotein diet and the unflavored control diet. Most of the rats either completely avoided the latter diet for the following 6 days as in row 2, Fig. 6, or selected only minor amounts of this nutritionally superior diet; thus, there were only 6 out of a possible 30 opportunities in 6 days (30 rat-days) when an individual rat selected any of the 8% amino acid control diet. Even when next offered a choice between the nonprotein diet and the familiar, anise-flavored control diet, the rats did not all choose the control diet (not shown). Following

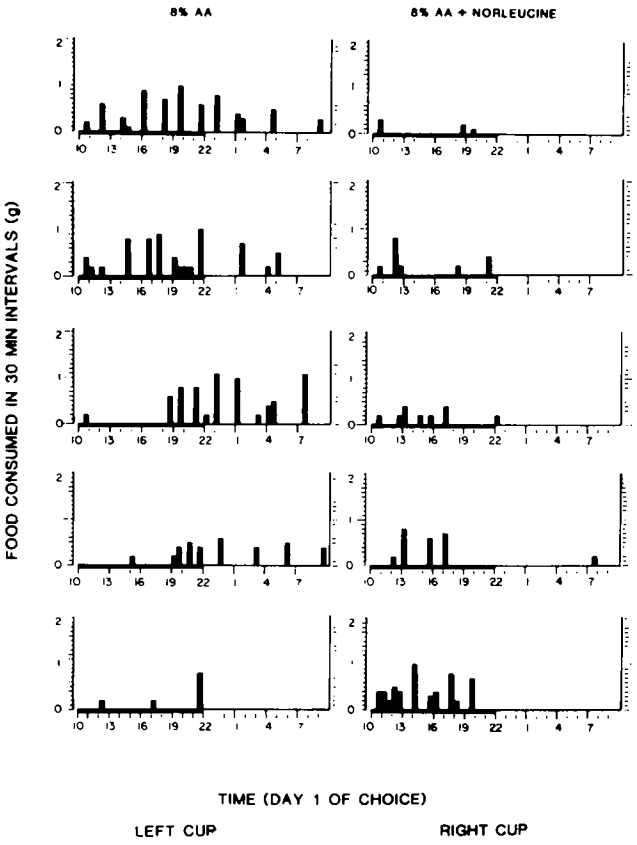

FIG. 4. Food selection during 30-min intervals on the first day of the choice by 5 rats between the leucine-limiting 8% amino acid control diet B and this diet containing norleucine (supplement 6, Table 1). Dark period is shown by heavy lines on abscissa. Each row shows the feeding pattern of a single rat. Diets were not flavored.

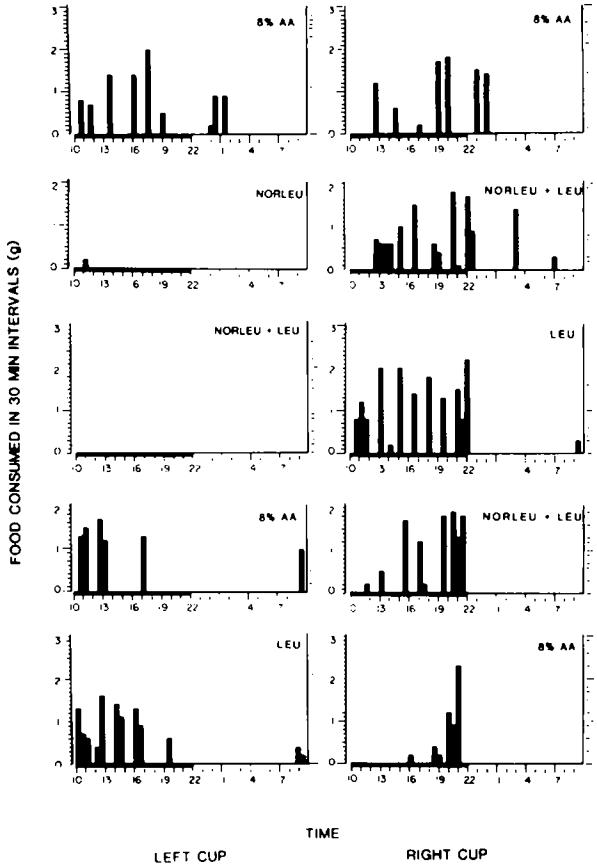

FIG. 5. Food selection during 30-min intervals by a single rat during the first day of succeeding choices from the unflavored diets indicated in the upper right of each section (each following a period of anise-flavored control diet B in both cups as in the top row); Norleu, supplement 6, Table 1; Norleu + Leu, supplement 7; Leu, supplement 1. Selection by this rat from the control and norleucine diets is shown in the bottom row of Fig. 4.

presentation of the unflavored control diet in both cups (row 3), the rats were again offered the choice between the nonprotein and unflavored control diets (row 4). All rats now sampled both cups and by the fifth day were uniformly preferring the control diet. Finally, 4 of the 5 rats selected more of the norleucine + leucine diet than of the nonprotein diet on the first day of this choice (row 5). Feeding patterns varied considerably during the following 2 days, but all rats preferred the amino acid-containing diet on the last 3 days of the choice.

In agreement with other reports (8, 13, 14) the results of Fig. 6 indicate that diet choices may be affected by preceding feeding experiences. Therefore, another choice study was performed in which a new group of rats received choices of Fig. 6 but in a different sequence. Figure 7A, which represents choices for 34 days by the rat of Fig. 6, shows that, after the rat had selected the nonprotein rather than the unflavored amino acid imbalanced diet (diets 2 and 3, days 3–6), it continued to prefer the nonprotein diet rather than the unflavored amino acid-containing control diet (diets 3 and 4, days 10–15), although the former diet will not permit growth. Addition of anise flavor did not change the choice of this rat, although some of the remaining rats did switch to the flavored control diet. Only after receiving the control diet alone (days 20–22) did the rat switch its preference to this better diet (days 23–28). In contrast, Fig. 7B shows that, with no prior experience with unflavored amino acid diets, the rat chose the amino acid control diet and avoided the nonprotein diet (diets 4 and 3, respectively; days 3–8). This rat also selected the diet containing norleucine + leucine (diet 5 vs. diet 3, days 11–14) and selected exclusively for 6 days the imbalanced diet containing norleucine (diet 2, days 15–20). All 5 rats preferred the norleucine + leucine diet during the 4-day choice period (days 11–14) and all consistently selected only the norleucine diet rather than the nonprotein diet during the first 2 days of that choice (days 15–16); 3 of the 5 rats maintained this choice throughout the 6-day period (days 15–20).

Analysis of feeding indices also showed strikingly different patterns of selection from the norleucine and nonprotein diets. In contrast to the results of Table 2B, no rat chose any of the nonprotein diet on day 1 of the choice (Table 2C); only 1 rat ate any of this diet on day 2 (0.1 g; not shown). The main difference in the patterns on day 6 was that average total intake of the norleucine diet during the dark was twice that of the nonprotein diet, and scarcely any feeding occurred during the light (2 meals by 1 rat; Table 2C). The 3 rats selecting the norleucine diet gained an average of 5 g during the 6-day period, while the 2 rats eating only the protein-free diet gained 0.5 g.

A possible complication in choice studies is that the rats often have position preferences. This is clearly illustrated in Fig. 7A, days 7–9 and 21–22, during which both food cups contained the

TABLE 2

CHARACTERISTICS OF CHOICES FROM DIETS WITH OR WITHOUT ADDED NORLEUCINE

Diet Pair	Total Intake (g)	Number of Meals	Meal Size (g)	Meal Duration (min)	IMI (min)	Rate (g/min)
A—Day 1						
8% AA						
dark (5)	3.0 ± 0.8	6.2 ± 1.4	0.47 ± 0.05	5.4 ± 0.7	110.7 ± 18.5	0.13 ± 0.01
light (4)	2.0 ± 0.7	3.8 ± 1.3	0.42 ± 0.11	5.4 ± 1.4	249.9 ± 118.6	0.08 ± 0.02
8% AA + NL						
dark (5)	2.3 ± 0.8	6.6 ± 1.7	0.34 ± 0.07	2.9 ± 0.9	107.3 ± 17.2	0.16 ± 0.01
light (2)	0.08 ± 0.05	0.4 ± 0.2	0.08 ± 0.05	0.4 ± 0.2	576.0 ± 88.2	0.10 ± 0.04
A—Day 2						
8% AA						
dark (5)	6.1 ± 1.1*	10.0 ± 2.0*	0.64 ± 0.06	7.5 ± 0.7*	68.5 ± 12.8	0.12 ± 0.01
light (5)	1.8 ± 0.6	3.0 ± 0.6	0.60 ± 0.13	7.7 ± 1.9	191.9 ± 26.9	0.12 ± 0.02
8% AA + NL						
dark (4)	0.9 ± 0.5*	2.0 ± 2.0*	0.29 ± 0.11	1.9 ± 0.8*	347.2 ± 105.0	0.17 ± 0.04
light (1)	0.04 ± 0.04	0.2 ± 0.2	0.04 ± 0.04	0.2 ± 0.2	648 ± 72	0.04 ± 0.04
B—Day 1						
0% AA						
dark (5)	5.4 ± 0.4†	15.8 ± 1.9†	0.36 ± 0.05	4.9 ± 0.9	41.4 ± 4.2†	0.09 ± 0
light (5)	0.8 ± 0.2	2.4 ± 0.9	0.43 ± 0.20	5.3 ± 2.0	266.3 ± 55.9	0.09 ± 0
8% AA + NL						
dark (5)	0.6 ± 0.3†	3.4 ± 0.5†	0.20 ± 0.08	2.8 ± 1.5	171.5 ± 20.2†	0.09 ± 0
light (3)	0.12 ± 0.07	0.8 ± 0.4	0.08 ± 0.04	1.0 ± 0.5	479.7 ± 100.6	0.06 ± 0.02
B—Day 2						
0% AA						
dark (5)	6.1 ± 0.1‡	15.4 ± 0.9‡	0.40 ± 0.03‡	5.3 ± 0.1‡	40.5 ± 2.8†	0.09 ± 0
light (5)	0.4 ± 0.1	2.2 ± 0.2	0.19 ± 0.05	2.1 ± 0.6	227.0 ± 12.3	0.10 ± 0.01
8% AA + NL						
dark (5)	0.2 ± 0.04‡	2.2 ± 0.4‡	0.10 ± 0‡	1.0 ± 0‡	240.0 ± 32.8†	0.10 ± 0
light (1)	0.02 ± 0.02	0.2 ± 0.2	0.02 ± 0.02	0.2 ± 0.2	648.0 ± 72	0.02 ± 0.02
C—Day 1						
0% AA						
dark (0)	0‡	0†	0†	0†	720 ± 0‡	0‡
light (0)	0	0	0	0	720 ± 0	0
8% AA + NL						
dark (5)	5.3 ± 0.4‡	7.2 ± 1.2†	0.80 ± 0.11†	6.8 ± 1.1†	91.2 ± 14.3‡	0.13 ± 0.01‡
light (3)	0.28 ± 0.17	1.0 ± 0.5	0.16 ± 0.08	1.5 ± 0.8	467.5 ± 108.2	0.06 ± 0.03
C—Day 6						
0% AA						
dark (3)[a]	2.6 ± 1.5	3.6 ± 2.2	0.39 ± 0.20	4.0 ± 1.8	389.8 ± 144.4	0.06 ± 0.03
light (1)	0.4 ± 0.4	0.4 ± 0.4	0.21 ± 0.21	2.3 ± 2.3	622.6 ± 97.4	0.02 ± 0.02
8% AA + NL						
dark (3)[a]	5.3 ± 2.2	6.4 ± 2.8	0.53 ± 0.28	4.4 ± 1.9	323.9 ± 161.8	0.07 ± 0.03
light (0)	0	0	0	0	720 ± 0	0

Meals represent the number of eating bouts separated by at least 5 min. Values are the mean ± S.E. for 5 rats; numbers in parentheses indicate those rats actually selecting at least once from the indicated diets. Means (without S.E.) for only those rats actually consuming food can be obtained by multiplying given values by 5/n (except for IMI which is 720 min in absence of food intake).

IMI, intermeal interval; NL, norleucine, Supplement 6, Table 1.

Amino acid diets are based on Diet B, Table 1.

Initial weights of rats in part A were 75.4 ± 0.5 g; part B, 80.2 ± 2.0 g; and part C, 94.6 ± 1.1 g.

[a]One rat ate only 0.3 g of the nonprotein diet while eating 8.6 g of the norleucine diet.

For dark period, *$p<0.05$; †$p<0.01$; ‡$p<0.001$; paired t-test.

anise-flavored control diet; at these times the rat ate little or nothing from its left food cup. A less obvious preference is also shown on days 9–10 in Fig. 7B. In order to avoid misleading results it is important that each of a given diet pair be offered an equal number of times in each position. Fortunately, position preferences appear to be readily overridden if the rats actually have a diet preference (Fig. 7). In contrast, when choices involved diets containing 0.2% norleucine, diet preferences were usually so weak

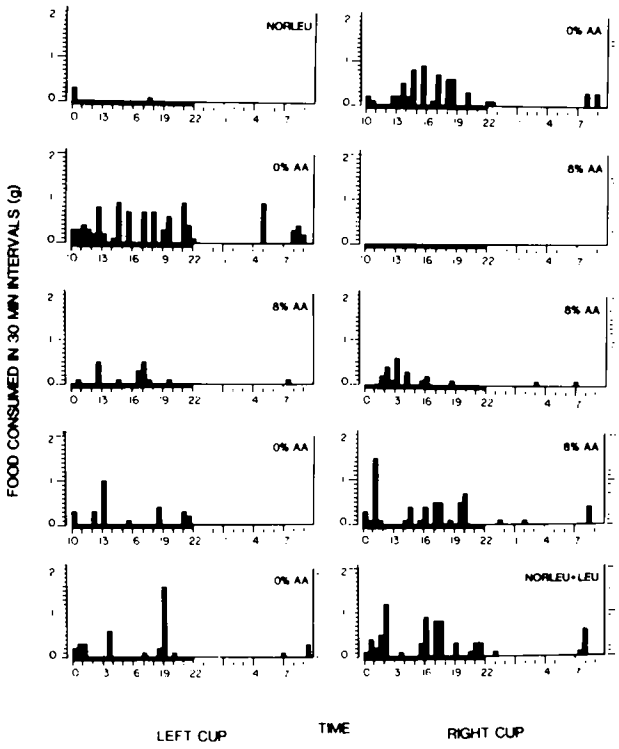

FIG. 6. Food selection during 30-min intervals by a single rat during the first day of succeeding choices from the unflavored diets indicated in the upper right of each section. Rats first received anise-flavored control diet B in both cups for 5 days before the choice shown in the top row. Norleu, supplement 6, Table 1; Norleu + Leu, supplement 7.

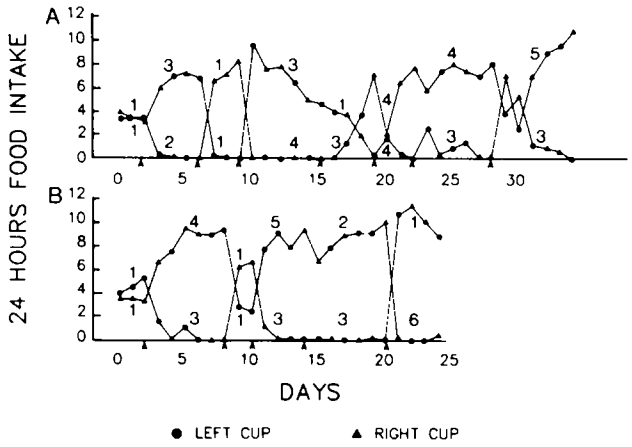

FIG. 7. Total daily food selection from succeeding diet choices by 2 individual rats. Section A shows grams of food selected by the rat described in Fig. 6; section B shows intakes by a rat offered the same diets as in section A but in a different sequence. Position of diet is shown by the symbols. Diets are identified by the numbers above the abscissas. Diet No. 1, amino acid control diet B flavored with 0.5% anise; No. 2, norleucine, supplement 6, Table 1; No. 3, protein-free; No. 4, unflavored control diet; No. 5, norleucine + leucine, supplement 7, Table 1; No. 6, protein-free + anise. Arrows on abscissa indicate the beginning of the first 24-hr period of the stated choice.

that position preferences were frequently apparent (not shown).

Amino Acid Concentrations

Concentrations of LNAA in plasmas and brains of rats in selected groups from Experiment 1 are shown in Table 3. These preliminary results show that the most striking changes occurred in the BCAA in both plasma and brain, with effects generally being more pronounced in brain than in plasma. Norleucine (Table 1, supplement 6) was highly effective in lowering brain leucine content; the rise in leucine content induced by additional dietary leucine alone (supplement 1) was so well blocked when the analog was included with leucine (supplement 7) that brain leucine concentration was only half the control value.

Dietary leucine or norleucine markedly reduced brain concentrations of isoleucine and valine, sometimes to undetectable levels which were equivalent to less than 5 to 10% of the control values (Table 3). Sums of concentrations of methionine, phenylalanine, tryptophan and histidine (ΣMPTH, Table 3) were unaffected in plasma but were low in brains of rats fed norleucine; the effects were much less striking than for the BCAA. Of these amino acids, only histidine was depressed in plasma (to 60% of the control value), suggesting that, after the BCAA, it may have become the next limiting amino acid in the diet; methionine, phenylalanine and tryptophan concentrations in brain were 80–85% of control values, while histidine was reduced to about 45% of the control when rats consumed diets containing norleucine alone.

Norleucine had generally little effect on concentrations in plasma and brain of amino acids other than the LNAA. Sums of concentrations of the cationic amino acids (ornithine, lysine and arginine) were not reduced; sums of concentrations of small neutral amino acids (threonine, serine, alanine and glycine) were reduced by less than 15% in brain only, primarily due to changes in threonine (results not shown). (Manuscript in preparation will include detailed results of amino acid changes in plasma, brain, liver and muscle.)

DISCUSSION

Early studies with the BCAA showed that racemic mixtures of leucine or isoleucine, but not of norleucine, served as indispensable amino acids for the rat (30). Instead, norleucine depressed growth when leucine was absent from the diet or present only in low amounts (16,30). The present studies show that dietary L-norleucine can produce distinct dose-related depressions in growth and food intake of young rats; thus, the originally described, adverse effects of norleucine are not due only to the D-isomer.

Our studies also demonstrate that norleucine was not directly toxic in these short experiments, because relatively large amounts of the other indispensable amino acids including leucine completely prevented the deleterious effects of the analog. The results also imply that the effects of norleucine were not restricted to interference with the BCAA, as dietary supplements of these amino acids alone were unable to completely reverse the effects.

Adaptation of rats to dietary norleucine occurred at the highest dose studied (1.1% of the diet), as there was recovery of early weight loss after the first few days of treatment; however, seldom was the rate of growth as great as that of the corresponding control rats. The degree of prevention of the norleucine effects depended on the dietary proportions of the other indispensable amino acids as well as on the leucine content of the diet. Thus, when these amino acids were not present in amounts adequate for optimal growth, a dietary excess of leucine did not completely prevent the growth depression induced by the highest level of norleucine. In contrast, such an excess of dietary leucine completely reversed the effects of norleucine when the other indispensable amino acids

TABLE 3

CONCENTRATIONS OF LARGE NEUTRAL AMINO ACIDS IN PLASMAS AND BRAINS OF RATS FED A LEUCINE-LIMITING DIET WITHOUT OR WITH ADDED LEUCINE OR NORLEUCINE

Treatment	Leucine	Isoleucine	Valine	ΣMPTH*	Norleucine†
			nmoles/ml plasma		
No Supplement	86 ± 3[a]	163 ± 16[a]	439 ± 35[a]	371 ± 38	—
Supplement 1	204 ± 17[b]	59 ± 5[b]	128 ± 17[b]	368 ± 25	—
Supplement 6	27 ± 2[c]	32 ± 4[c]	79 ± 7[c]	362 ± 39	697 ± 50
Supplement 7	84 ± 15[a]	30 ± 4[c]	68 ± 10[c]	332 ± 30	627 ± 70
			nmoles/g brain		
No Supplement	47 ± 1[a]	74 ± 5[a]	249 ± 24[a]	239 ± 16[a]	—
Supplement 1	82 ± 3[b]	13 ± 3[b]	17 ± 4[b]	221 ± 3[a]	—
Supplement 6	9 ± 2[c]	5 ± 2[b,c]	ND[c]	167 ± 4[b]	340 ± 11
Supplement 7	22 ± 4[d]	ND[c]	ND[c]	139 ± 6[c]	290 ± 9

Rats were fed control diet A, Table 1; results were selected from those for rats of Fig. 1 (Experiment 1). For supplement description see Table 1, Experiment 1.

*Sum of concentrations of methionine, phenylalanine, tryptophan and histidine.

†Includes tyrosine which coelutes with norleucine; tyrosine is probably less than 20% of the total value (23,24).

Different superscript letters indicate significant differences ($p<0.05$; ANOVA followed by the LSD test).

ND: Nondetectable (valine <5 nmoles/g brain; isoleucine, <3 nmoles/g).

were also present in slight excess. Although leucine was always present in the control diets in the same amount (65% of the requirement for growth), the severity of the norleucine-induced growth depression was greater when the amounts of the other indispensable amino acids in the diet were higher, suggesting that the efficiency of leucine utilization was increased in rats receiving slightly excessive amounts of the other amino acids. Thus, leucine effectively became even more limiting under these conditions. Also, high dietary levels of the indispensable LNAA may contribute to the competition by norleucine for leucine transport into various tissues, as suggested by other studies in which inclusion of norleucine in a low protein diet caused distinct reductions in concentrations of large neutral amino acids, especially of the BCAA, in brain and muscle (24). Calculation of the distribution ratios for the BCAA of Table 3 (the ratios of brain to plasma concentrations) showed that dietary norleucine clearly reduced the amounts of these amino acids in brain relative to those in plasma. Ratios were reduced from 0.55, 0.45 and 0.57 for leucine, isoleucine and valine, respectively, for the control rats to 0.33, 0.16 and 0.06 for the rats fed norleucine at 1.1% of the diet. Ratios for the remaining LNAA were much less affected than were those for the BCAA.

The diet choice experiments show that rats avoided a leucine-limiting, amino acid diet in which an antagonism was created by adding norleucine, a strong competitor for transport of natural LNAA across the blood-brain barrier. If this diet also contained additional leucine the rats no longer consistently avoided it, even if the alternate diet did not contain norleucine. Thus, the adverse physiological effects induced by norleucine alone (reduced food intake or diet avoidance) were presumably prevented when leucine was also present in the diet. These observations are consistent with those of Leung and associates (12,14) who studied an amino acid disproportion involving threonine, although composition of their diets differed considerably from ours. In their experiments the disproportion was produced by adding a mixture of all of the indispensable amino acids except threonine to a low protein diet limiting in that amino acid (threonine imbalance). Their computer-monitored feeding patterns showed that, after the initial 12-hr light period during which the rats consumed numerous, approximately 0.05 g meals from the imbalanced diet, they clearly avoided the imbalanced diets within 2 to 3 hr after the beginning of the first dark period and selected many large meals from the control diet alone (12). In general, our rats clearly began to avoid the norleucine-containing diet and to prefer the control diet within 2 to 9 hr after the diet was first presented at the beginning of the dark period; only one rat took as long as 12 hr to make this choice (Fig. 4). This preference occurred primarily because of a 5-fold increase in the number of modestly larger meals. The rats ate little or none of the norleucine diet during the last half of the first day (light period). It is not clear if these relatively rapid choices reflect a more severe dietary disproportion than that of Leung *et al.* (12) or if the rats, preferring to eat during the dark, simply ate during the early stages of this period enough of the diet to permit rapid development of the adverse effects leading to altered choice. The extent of sampling from different diets before a preference is established may be inversely related to the relative degree of aversion induced by each diet when fed alone.

Early observations showed that rats select a nonprotein diet rather than one containing disproportionate amounts of amino acids (8,14). More recently Leung *et al.* (12) found that rats ate many small meals from the threonine-imbalanced and nonprotein diets following initial exposure during the first light period, but did not make a choice between these diets until the following dark period. Our rats clearly began to prefer the nonprotein rather than the 1.1% norleucine diet within 1 to 6 hr after the choice first became available (dark period). Although Leung *et al.* found that rats would avoid the nonprotein diet and select the familiar threonine-limiting control diet during the first day of choice, our rats continued during a 6-day period to select almost exclusively the nonprotein rather than the leucine-limiting control diet. A possible explanation is that our rats had been originally fed the control diet flavored with anise; therefore, this same diet without anise may have been undetectably different in taste and odor from the unflavored norleucine diet already recognized by the rats to be unacceptable. However, not all the rats preferred the anise-flavored control diet to the nonprotein diet when this choice

followed that between the nonprotein and unflavored control diets.

It is evident that prior experience can alter subsequent diet choices (14); rats in our studies almost completely avoided the unflavored control diet in favor of the nutritionally inadequate nonprotein diet until after they were fed only the former diet. A more striking example is the finding that, if the rats were first exposed to the unflavored control diet or this diet containing norleucine plus leucine (relatively nonaversive diets), they subsequently chose the unflavored, norleucine-containing diet over the nonprotein diet. This choice, which occurred essentially without exception during the first 2 days, was directly opposite to that made when the first exposure was to the unflavored norleucine and nonprotein diets. These observations suggest that addition of a flavoring agent may help disguise the possibility that unflavored and flavored diets are otherwise identical. As another example, on the first day of the choice between unflavored control and norleucine diets, rats clearly ate less of the unflavored control diet than of the equivalent flavored diet available on the preceding day.

Taste does not seem to be an important explanation for avoidance of certain diets in these choice studies; depending on the circumstances, rats may select a diet that is avoided at other times. Thus, Fig. 5 shows both complete avoidance and preference for the diet containing norleucine plus leucine. Also, rats which normally avoided a GABA-containing diet selected this diet if the alternate choice was made sufficiently aversive (26). Leung *et al.* (11) concluded that, although taste may alter diet choices, this effect can be overridden by exposing the animal to the metabolic effects of either a beneficial or aversive diet. Furthermore, rats actually prefer a norleucine solution to water (27) at concentrations of norleucine similar to those in the dry diets which were avoided in the present studies.

Interpretation of diet choice studies may be compromised by factors such as location of food and by individuality of rat behavior. The former problem can be lessened by rotating diets equally among the available feeding locations. Individual behaviors seem most likely to occur when choices are between diets having similar aversive qualities or when neither diet has a clearly undesirable physiological effect. Such individuality suggests that rats have different metabolic responses or thresholds to a given dietary component.

These and earlier observations show that, when rats are fed diets containing amino acid analogs such as norleucine (24) or homoarginine (24, 25, 28), patterns of growth and feeding behavior and responses in amino acid pools resemble those observed when rats are fed diets with disproportions of natural amino acids; these have been categorized, operationally, as amino acid imbalances, antagonisms and toxicities (8). "Imbalance" has been used to characterize diets containing moderate surpluses, usually of several indispensable amino acids, which result in depressions of food intake and growth that are prevented by a supplement of the amino acid limiting in the diet. The present experiments are examples of this category. "Antagonism" has been restricted to situations in which a dietary surplus, usually of a single amino acid, causes depressions in growth and food intake that are prevented by small supplements of one or more structurally similar amino acids, e.g., leucine, isoleucine and valine, or lysine and arginine. "Toxicity" has been applied to effects of large dietary surpluses of individual amino acids that do not fit the definition of imbalance or antagonism, even though in many cases the effects consist only of food intake and growth depressions; only a few amino acids, e.g., methionine and tyrosine, produce clearcut toxic reactions. These categories, which have many features in common, were established before it was widely recognized that diet-induced disproportions of amino acids in the blood have the potential to modify competition for transport into tissues, especially brain, and thereby to alter amino acid pools (7,15). This realization has led to a new and major direction in investigations of the effects of altered dietary amino acid patterns.

Although the selective depression in brain of the concentration of a given amino acid may be associated with depressed food intake, this effect is not necessarily directly dependent on depleted brain pools. Calculation of the correlation coefficient from results of all of the groups from the present experiments showed that food intake during the last day of the 10-day experimental periods was strongly correlated with brain leucine concentration ($r = .61$; $p<0.01$; $n = 36$). There was also a moderate correlation of food intake with brain concentration of leucine when the calculation was based on means for only those groups of rats receiving norleucine [$r = .41$; $n = 22$; for $p<0.05$, $r = .41$ (20)]. Sums of the BCAA concentrations in brain were not correlated with food intake ($r = -.11$; $n = 22$) because valine and isoleucine concentrations sometimes were extremely low without accompanying reductions in food intake. Brain norleucine content and sums of concentrations of the remaining LNAA in brain (MPTH) also were not correlated with food intake ($r = -.18$ and $-.002$, respectively; $n = 22$). Rats presumably regulate their food intake by some neural means; amino acids may be important under specific conditions as precursors for synthesis of a critical neurotransmitter(s), peptide(s) or protein(s).

Our studies with agents such as norleucine suggest that amino acid analogs generally can be useful tools, not only in investigations of amino acid metabolism and function (2), but also in studies of alterations in animal feeding behavior associated with changes in brain amino acid pools, as observed with all of the categories of dietary disproportions of amino acids. Analogs such as norleucine, norvaline and homoarginine do not appear to be metabolized to toxic intermediates (4, 5, 18, 29), are not normally incorporated into mammalian proteins, compete readily and effectively with naturally occurring amino acids for uptake into brain and, in moderate amounts in the diet, cause changes in feeding behavior resembling those observed with disproportions of naturally occurring amino acids. Use of selected analogs would appear to provide specific models for investigating features of various types of dietary amino acid disproportions, while avoiding some of the complications that arise from metabolic interactions when the natural amino acids are used (9).

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