Effect of Protein–Calorie Restriction on Brain Amino Acid Pool in Neonatal Rats[1] (35403)

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There is increasing interest in the effect of early nutritional deprivation on the subsequent development of the nervous system. Severe undernutrition during the early part of life, when brain growth is most rapid, results in a permanent reduction in brain size and in a related restriction of behavioral development both in humans and in experimental animals (1–5). Undernutrition during the preweaning period resulted in deficits in brain weight and lipid, phospholipid, cholesterol, cerebroside, and DNA content of brain, and in decreased synthesis of sulfatide; subsequent *ad libitum* feeding had no significant effect in restoring the chemical composition of the brain (6–11).

However, the relationship between protein–calorie malnutrition or calorie malnutrition and protein synthetic activity of the brain and the composition of its free amino acids pool has not received much attention. Free amino acids are not only the precursors for proteins and neuromediators but also are involved in many biosynthetic and energy-producing reactions (12). These processes require a continuous supply of free amino acids into the system. Although the distribution and levels of amino acids in the brain are undoubtedly controlled by a "blood–brain barrier system" which includes organ membranes, as well as cell membranes and membranes of particulates within the cells, it has been shown that there is a rapid exchange between plasma and brain amino acids with half-life times of most cerebral amino acids in minutes (13). The concept of a very active ribosmal system taking up amino acids and synthesizing protein which is then transported along the axon to the synapse, where it plays an important functional role, also suggests a strong dependence on a supply of amino acids from the blood stream (14). This, in turn, depends on an adequate quantity and quality of dietary proteins and seems most critical at the age when the central nervous system is in a stage of rapid development. What little work has been conducted, in relation to the effect of protein malnutrition on brain protein metabolism, was done with adult rats. Feeding of either protein-free diet or protein-deficient diet to adult rats has no effect on protein content, free amino acids, and DNA content of the brain (15) or on its protein synthetic activity (16) indicating that once the brain has attained its adult size, it is resistant to nutritional damage.

Recent reports have emphasized the importance of chemically defined, water-soluble, antigen-free diets and germfree animals in nutritional and immunological studies (17, 18). Nutritional adequacy of this diet has been shown by the fact that the germfree mice were able to reproduce into fifth generation and germfree rats were able to grow normally. When this diet was hand-fed to newborn germfree rats which had never nursed their mothers, the animals seemed incapable of taking in the diet in sufficient quantities without distention and eventual rupture of gut. It is possible that this restricted intake during early postnatal development, when the central nervous system is in a stage of rapid development, might affect the supply of amino acids to the brain for normal protein synthesis and other energy-producing reactions.

The present study was designed to elu-

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cidate the possible effects of restricted calorie intake (protein–calorie malnutrition) during early postnatal development on the composition of free amino acid pool of brain in rats.

Materials and Methods. Germfree rats of Lobund stock (Wistar origin) were used. The use of only germfree rats was required because when experiments with infant rats fed water-soluble diet were conducted in an open environment, death caused by intestinal invasion of yeasts was a common occurrence. Pregnant germfree females were housed individually in stainless steel cages with nesting material and were fed L-485 diet (19). Immediately after parturition, one group of litters were allowed to suckle their mothers normally and another group was separated from the mother and hand-fed limited quantities of Millipore-filtered chemically defined water-soluble diet, L-487WS-2, and fat and fat-soluble vitamin supplement (Table I) for 2 days. The amino acid composition of this diet was formulated on the basis of rat milk and carcass analysis. In this series, baby rats hand-fed sufficient calories were not included because the gut of newborn rat appeared not to accept such amounts without distention and eventually rupture. The mother-fed and hand-fed baby rats consumed about 2.40 and 0.68 calories/day, respectively (21). Then the baby rats were killed by ether anesthesia. The spinal cord was transected just below the level of calamus scriptorius and the brain was extirpated as quickly as possible, weighed, and frozen immediately between two pieces of dry ice.

The free amino acids and other easily extractable ninhydrin-positive substances were extracted from the individual brains according to the methods of Stein and Moore (22), and Tallan *et al.* (23) with slight modifications. The brain tissue with an added amount of norleucine solution as an internal standard, was homogenized with 15 vol of cold 1% picric acid in a Potter-Elvehjem homogenizer cooled with crushed ice. The precipitate was centrifuged down and washed twice with 0.1 *N* HCl. From the combined supernatants, the picric acid color was removed on a small column of Rexyn 201, 200–400 mesh in chloride form (Fisher Scientific Co., Chicago). The column was washed three times with 4-ml portions of 0.02 *N* HCl. The colorless eluate and acid washings were concentrated in a rotary evaporator to about 6 ml, adjusted to pH 7.5, and allowed

TABLE I. Composition of Water-Soluble Diet, L-487WS-2 Fed to Baby Rats.[a]

Ingredients	(g/100 g of fat-free diet)
L-Leucine	6.33
L-Phenylalanine	2.64
L-Isoleucine	3.43
L-Methionine	3.43
L-Tryptophan	1.32
L-Valine	2.64
Glycine	1.05
L-Proline	5.27
L-Serine	4.74
L-Asparagine	3.69
L-Arginine HCl	2.90
L-Threonine	2.64
L-Lysine HCl	6.33
L-Histidine HCl • H_2O	2.64
L-Alanine	2.11
Sodium L-glutamate	12.12
L-Tyrosine ethyl HCl	2.64
Dextrose	5.27
Lactose	5.27
Ferrous gluconate	0.105
Monocalcium fructose 1,6-diphosphate	12.39
$CaCl_2 \cdot 2H_2O$	3.16
$MgCl_2 \cdot 6H_2O$	3.95
NaCl and KI mixture (125:1)	0.184
CH_3COOK	2.79
Trace mineral mixture-35[b]	0.163
Choline chloride	0.659
B-vitamin mixture-111E2[c]	0.171
Ladek-68E2 (fat and fat-soluble vitamins)[d]	—

[a] L-487WS-2 is a modification of diet reported in (17). Each baby rat was fed 60 mg of fat-free solids and 50 mg of fat and fat-soluble vitamin supplement (Ladek-68E2)/day.

[b] Trace mineral mixture contained (mg): $Mn(C_2H_3O_2)_2 \cdot 4H_2O$, 118; $ZnSO_4 \cdot H_2O$, 29; $Cu(C_2H_3O_2)_2 \cdot H_2O$, 7.9; $Cr(C_2H_3O_2)_3 \cdot H_2O$, 6; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.79; NaF, 1.29; Na_2SeO_3, 0.05; $Co(C_2H_3O_2)_2 \cdot H_2O$, 0.24.

[c] For detailed composition see Ref. (20).

[d] Ladek-68E2 was administered separately (50 mg/day/animal). Daily supplement contained: cholesterol, 100 μg; vitamin A palmitate, 0.32 μg; vitamin K_1, 51 μg; vitamin D_3, 0.001 μg; *dl*-α-tocopherol acetate, 29.2 μg; *dl*-α-tocopherol, 14.6 μg; ethyl linoleate, 1.456 mg; corn oil, 48.35 mg.

to remain at room temperature for 4 hr to convert cysteine to cystine. The samples were then evaporated to a small volume in a freeze drier, adujsted to pH 2.0 with 1 *N* HCl and made to volume with pH 2.0 citrate buffer. Triplicate determinations were made on each sample. The amino acids and other Ninhydrin-positive compounds were separated with Technicon Automatic Amino Acid Anlayzer, Model TSM (Technicon Corp., Tarrytown, New York). The individual peaks were quantitated using an integrator.

Results and Discussion. Restriction of protein–calories to baby rats during the first 2 days of life caused a depression of body weight (4.40 vs 5.25 g) and size of the brain (0.26 vs 0.31 g) compared to those allowed to suckle their mothers normally.

Brain free amino acid pattern in baby rats suckled normally or fed restricted calories is shown in Table II. The concentration of certain essential amino acids, particularly valine, leucine, isoleucine, histidine, and arginine, was significantly increased in baby rats fed restricted calories compared to those suckled normally. No difference in levels of threonine between the groups was found. Baby rats maintained on restricted calories showed a decline in certain nonessential amino acids, especially glutamic acid, glutamine, alanine, and serine, and also GABA. Cystine level was slightly but significantly elevated in baby rats fed restricted calories. There was no difference in aspartic acid and glycine levels between the groups. Ornithine, urea, and NH_3 were significantly increased in those rats fed restricted calories for a short period.

TABLE II. Free Amino Acids in Brains of 2-Day-Old Rats Suckled Normally (Group 1) or Fed Restricted Calories (Group 2).

Amino acids	Group 1[a] (μmoles/g)	Group 2[a] (μmoles/g)
Essential		
Threonine	0.58 ± 0.04	0.58 ± 0.03
Lysine	Trace	Trace
Valine	0.32 ± 0.07[b]	0.50 ± 0.02
Leucine	0.22 ± 0.02[b]	0.78 ± 0.01
Isoleucine	0.15 ± 0.01[b]	0.45 ± 0.11
Phenylalanine	Trace	Trace
Histidine	0.14 ± 0.08[b]	0.63 ± 0.10
Arginine	0.05 ± 0.002[b]	0.17 ± 0.004
Nonessential		
Aspartic acid	1.83 ± 0.24	2.42 ± 0.16
Glutamic acid	5.12 ± 0.26[b]	3.49 ± 0.18
Glutamine	5.84 ± 0.54[b]	3.59 ± 0.48
Alanine	1.10 ± 0.09[b]	0.39 ± 0.15
Glycine	1.80 ± 0.05	1.60 ± 0.08
Serine	0.88 ± 0.03[b]	0.76 ± 0.03
Tyrosine	0.11 ± 0.06	0.20 ± 0.02
Cystine	0.53 ± 0.02[b]	0.67 ± 0.03
Other ninhydrin-positive compounds		
o-Phosphoethanolamine	3.11 ± 0.15	3.49 ± 0.11
Taurine	10.67 ± 1.13	10.56 ± 0.22
γ-Aminobutyric acid	1.65 ± 0.10[b]	1.05 ± 0.08
Ornithine	0.025± 0.002[b]	0.04 ± 0.002
Ammonia	1.48 ± 0.10[b]	3.22 ± 0.13
Urea	5.70 ± 0.85[b]	15.03 ± 1.57

[a] Averages of 5 animals in group 1 and 4 in group 2 with triplicate determinations on each animal ± SEM.

[b] Significantly different from group 2, $p < 0.05$.

Since the data suggest that protein–calorie deficiency even for a short period during neonatal development altered the composition of the brain free amino acid pool in rats, increasing certain essential amino acids and decreasing certain nonessential amino acids, it is pertinent to elaborate on the possible mechanisms involved and also its impact on normal protein synthesis. Several of the most likely hypotheses to account for altered composition of the free amino acid pool have been proposed under our experimental conditions.

a. The first possibility is that alteration in the composition of free amino acid pool might have resulted from the dietary imbalance of one or more amino acids. This seems improbable in view of the fact that germfree mice were able to reproduce into the fifth generation on a similar diet fed *ad libitum* (17) indicating qualitative adequacy of this diet. Moreover, diet, L-487WS-2 was formulated on the basis of rat milk and carcass analysis.

b. Another possibility is the influence of physical characteristics of the water-soluble diet on the absorption. It is probable that the amino acids of the water-soluble diet, requiring no digestion, are absorbed directly upon entering duodenum, and also possible that

some individual amino acids are absorbed much later than the others so that there is a temporary imbalance of amino acids in the blood. However, the rats were fed every hour and the absorption would even out with time.

c. A third possibility is that under protein–calorie deficiency there is a preferential utilization of amino acids for energy purposes compared to synthesis of proteins. As a result, improper proportions and quantities of amino acids may be available for protein synthesis. It has been reported that protein synthesis in the brain is dependent upon the presence of amino acids in adequate amounts and in proper proportions (24–26). This could account for alteration in the composition of free amino acids in the brain of infant rats under protein–calorie deficiency. Relatively increased levels of ornithine, urea, NH_3, and arginine in the brains of rats fed low calorie diet might have resulted from increased catabolism of various amino acids via the urea cycle to provide energy.

d. In view of the fact that a decrease in concentration of certain nonessential amino acids and an increase in certain essential amino acids might also result from a reduced conversion of essential amino acids to nonessential amino acids, it is possible that this decrease in conversion might be due to deficiency in certain enzymes related to amino acid metabolism such as transaminases, dehydrogenases, and decarboxylases. This interpretation would agree with the findings of Rajalakshmi *et al.* (27) that there was a deficit of certain brain enzymes associated with amino acid metabolism in rats fed on a low protein diet.

Although the present study does not show conclusively that malnourished neonatal rats have deficiency in brain protein synthesis, the evidence presented indicates that the alteration of amino acid pool in the brains of malnourished rats might affect brain protein synthesis. Further studies on the effect of protein–calorie malnutrition on brain protein synthetic activity at subcellular level in terms of changes occurring in polysomal and oligosomal patterns, free ribosomes and ribosomal subunits, and in terms of amino acid-incorporating activity of polysomes. On this basis, it is also necessary to carry out further experiments to test whether these effects are irreversible upon subsequent rehabilitation. This approach makes it meaningful to correlate the effects of protein–calorie malnutrition at various periods of development, in terms of its protein biosynthetic activity, with the related restriction of intellectual or behavioral development as observed by various investigators.

Summary. Newborn rats fed restricted calories during the first 2 days of life showed a depression of brain weight, and the levels of glutamic acid, glutamine, alanine, serine, and GABA compared to those allowed to suckle their mothers normally. Also it was found that the levels of valine, leucine, isoleucine, histidine, arginine, cystine, ornithine, urea, and ammonia were increased in newborn rats maintained on restricted calories. It is concluded that calorie malnutrition, even for a short period, during the neonatal development is associated with alteration in the brain amino acid pool.

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