Influence of High Concentrations of Phenylalanine on the Amino Acids of Cerebrospinal Fluid and Blood.* (31306)

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Previous acute and chronic studies of experimental animals with high blood phenylalanine (PA) concentrations have indicated a striking competitive inhibition of the net transport of certain essential amino acids from blood to brain(1,2). A 50% reduction of the intracerebral pools of free valine, methionine, leucine, and isoleucine was found. In the present investigations we sought to determine whether the human cerebrospinal system would reflect changes similar to those observed in the brains of experimental animals, when blood PA was elevated.

A low-PA synthetic diet of known composition was administered to phenylketonuric subjects, and the inclusion or omission of an L-phenylalanine supplement was made the critical variable. The effect on amino acid concentrations in serum and CSF was determined.

Experimental. Six phenylketonurics, ranging in age from 6-17 years and including one female, were maintained on a diet in which a casein hydrolysate (Lofenalac®) provided the sole source of protein. The hydrolysate was mixed in a vehicle of fruit or corn starch pudding which provided the only extrinsic sources of calories or proteins. Once taste preferences were established prior to the experimental period, the vehicle and the amount of hydrolysate (supplemented or unsupplemented) remained constant. The fixed intakes which continue to the present were originally established on the following basis:

1. Calories: 62 cal/K/da.
2. Proteins: 1.54 g/K/da.
3. Nitrogen: 243 mg/K/da. (low phenylalanine diet).
   254 mg/K/da. (high phenylalanine diet).

In the individual subject the average daily intake of the synthetic diet varied less than ± 4% during the 2 weeks preceding each sampling of blood and CSF.

Mean weight gain was maintained at the rate of approximately 0.15 K/week during the experimental periods and has continued at that rate for a year following these studies. This suggests that the diet was more than ample to satisfy metabolic and appetite requirements. Furthermore, hemoglobin, packed red cell volume, total protein, and cholesterol have remained relatively unchanged in the normal range throughout the experimental periods and during subsequent months.

Fasting specimens of blood and CSF were obtained under the following conditions: first, when the blood PA was low; and then 4-5 weeks after addition of an L-phenylalanine supplement (1.9 g per 100 g of Lofenalac®), an amount sufficient to produce a high plasma concentration of the added amino acid. In the first experiment blood and CSF from each subject, at each PA level, were deproteinized, paired, and analyzed immediately. In the replication of this experiment the specimens were frozen within one-half hour after they were obtained and kept at −20°C, and the preliminary specimens of blood and CSF paired with the respective fluid withdrawn one month later. The two procedures showed no significant differences which might have suggested deterioration during storage. In addition 3 phenylketonuric retardates in the same age range on unrestricted diets were included for purposes of comparison.

Serum was deproteinized with an equal volume of 6% sulfosalicylic acid, and CSF, by addition of 60 mg of solid sulfosalicylic acid to 2 ml of spinal fluid. Supernatant fluids from the serum or CSF, 0.5 ml or 1.5 ml, respectively, were applied to twin chromatographic columns (I.D. 0.6 cm) and analyzed simultaneously. The techniques were

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Amino Acid Concentrations of Phenylketonurics on Low and High Phenylalanine Diets.

TABLE I. Cerebrospinal Fluid

	Unrestricted†	Low PA‡	High PA‡	%§
Essential amino acids (mg %)				
Phenylalanine	4.040– 9.680	.173 ± .11	9.74 ±3.4	563‖
Tyrosine	.117– .250	.089 ± .023	.236 ± .058	265‖
Threonine	.189– .311	.530 ± .13	.262 ± .28	49‖
Histidine	.220– .513	.276 ± .057	.383 ± .073	139‖
Methionine	—	.052 ± .015	.071 ± .017	137‖
Tryptophan*	—	.0497± .017	.0998± .014	201‖
Valine	.085– .125	.154 ± .028	.158 ± .053	103
Isoleucine	—	.038 ± .009	.043 ± .013	113
Leucine	.052– .057	.100 ± .029	.094 ± .024	94
Lysine	.135– .396	.306 ± .057	.304 ± .040	99
Non-essential amino acids (mg %)				
Alanine	.108– .135	.301 ± .052	.276 ± .063	92
Arginine	.153– .280	.296 ± .066	.230 ± .047	78‖
Aspartic acid	—	.0044± .001	.0053± .001	120
Citrulline	—	.032 ± .012	.047 ± .017	147‖
Glutamic acid	.110– .204	.256 ± .118	.323 ± .167	126
Glutamine	4.099–11.390	4.53 ±1.27	6.12 ±1.60	135‖
Glycine	.026– .047	.067 ± .015	.069 ± .031	103
Ornithine	—	.050 ± .017	.046 ± .011	92
Serine	.215– .527	.441 ± .07	.438 ± .08	99
Carnosine	—	2.91 ± .75	6.00 ±1.20	206‖

TABLE II. Serum

	Unrestricted†	Low PA‡	High PA‡	%§
Essential amino acids (mg %)				
Phenylalanine	27.860–31.980	1.00 ± .67	42.84 ±7.7	4,284‖
Tyrosine	.684– .773	.61 ± .15	.72 ± .29	118
Threonine	.146– 1.714	2.68 ±1.30	1.44 ± .28	54‖
Histidine	1.270– 1.599	1.88 ± .33	1.06 ± .30	56‖
Methionine	.256– .328	.40 ± .05	.44 ± .09	110
Tryptophan*	—	1.09 ± .27	1.23 ± .27	113
Valine	1.638– 2.060	2.35 ± .38	2.51 ± .68	107
Isoleucine	.376– .507	.51 ± .07	.56 ± .17	110
Leucine	.869– 1.100	1.12 ± .22	1.00 ± .27	89
Lysine	2.519– 3.368	2.72 ± .56	2.53 ± .42	93
Non-essential amino acids (mg %)				
Alanine	1.532– 3.619	3.58 ± .57	2.80 ± .70	78‖
Arginine	1.306– 1.955	1.68 ± .33	1.52 ± .25	91
Aspartic acid	.232– .521	.144 ± .06	.102 ± .04	71
Citrulline	.405– .682	.43 ± .19	.48 ± .14	112
Glutamic acid	.987– 2.744	.79 ± .19	.61 ± .25	77
Glutamine	4.220– 5.845	8.01 ± .81	6.67 ±1.28	83
Glycine	2.133– 2.794	1.88 ± .94	1.68 ± .52	89
Ornithine	1.100– 1.327	.65 ± .15	.56 ± .14	86
Serine	2.024– 2.240	2.18 ± .23	1.56 ± .22	72
Carnosine	—	—	—	—

TABLE III. Serum/CSF Ratios

	Low PA‡	High PA‡		Low PA‡	High PA‡
Essential amino acids (mg %)					
Phenylalanine	5.8	4.5	Tryptophan*	21.9	12.3‖
Tyrosine	6.8	3.1‖	Valine	15.3	15.9
Threonine	5.1	5.5	Isoleucine	13.4	13.0
Histidine	6.8	2.8‖	Leucine	11.2	11.1
Methionine	7.7	6.2	Lysine	8.9	8.3

TABLE III. Continued

	Low PA‡	High PA‡		Low PA‡	High PA‡
Non-essential amino acids (mg %)					
Alanine	15.9	13.3	Glutamine	2.1	1.3‖
Arginine	5.7	6.6	Glycine	36.9	41.0
Aspartic acid	32.7	19.2‖	Ornithine	13.0	12.2
Citrulline	13.4	10.2	Serine	4.9	3.6
Glutamic acid	3.1	1.9‖			

* Fluorimetric assay(5).
† Range of concentrations for 3 phenylketonurics on unrestricted diets.
‡ Mean of 6 PKU subjects ± S.E.D. from replicated studies.
§ Changes in concentrations expressed as a percentage of the values on the low phenylalanine diet.
‖ P .05-.01.

similar to those used by Piez and Morris(3) employing gradient elution, as modified by Efron(4). The pH gradient was produced by loading an Autograd (Technicon) with 150 ml of pH 2.875 buffer in the first 5 cells, 150 ml of pH 4.750 buffer in the last 3 cells, and 30 ml of pH 2.875 and 120 ml of pH 4.750 in cell #6 which is between. The columns were maintained at 37°C for the first 2¼ hours with the remaining 19 hours at 50°C. The spectrophotofluorimetric method of Hess and Udenfriend(5) was used for analyzing tryptophan because of its greater sensitivity in the range of concentrations found in blood and CSF.

Results. The concentrations of 10 essential amino acids (including tyrosine) and 9 non-essential amino acids were compared under conditions of normal and elevated circulating PA in cerebrospinal fluid (Table 1) and serum (Table II). Following the increase in PA, amino acid concentrations in the cerebrospinal system fell in only 2 instances: threonine and arginine decreased to 49% and 78% of their original values, respectively (Table I). Four essential amino acids (*viz.*, tyrosine, tryptophan, methionine, and histidine) and 2 non-essential acids (citrulline and glutamine) showed notable increases and the remainder were unchanged. Carnosine, which is a dipeptide of histidine and β-alanine, also increased.

In serum, decreases occurred in threonine, histidine, and alanine; phenylalanine was the only amino acid to rise (Table II). A 50% decrease in the serum concentration of threonine corresponded to its decrease in CSF.

Particularly striking is the lack of change in serum:CSF ratios for leucine, isoleucine, valine, and methionine, which had previously shown significant *rises* in blood:brain ratios under conditions of PA-loading in experimental animals(1,2). In our human subjects a rise in blood phenylalanine resulted in a *decrease* in the serum:CSF ratios of histidine, tryptophan, and tyrosine among the essential group. In contrast, PA itself and threonine, in spite of highly significant alterations in their absolute values, maintained the same ratios under conditions of high- and low-PA concentration. Among the non-essential amino acids the ratios of glutamine, glutamic acid, and aspartic acid, all active in the energy metabolism of brain, fell proportionately to about 4/7 of their original values, reflecting the increased CSF concentrations of these amino acids with high PA levels.

It will be noted that in general the range of amino acid concentrations in the sera and CSF of the 3 phenylketonurics on unrestricted diets indicated considerable variability both among themselves and when compared with those subjects on synthetic diets with added phenylalanine.

Discussion. It is apparent that the relationship of amino acid concentrations in the blood and CSF of human phenylketonurics bears little correspondence to the blood-brain relationships found in rodent species with experimentally elevated concentrations of PA.

The changes in amino acid concentrations which took place in human CSF in the presence of high circulating levels of PA tend to

be, if anything, opposite from those found under similar conditions in rat brain. In rat brain the concentrations of leucine, isoleucine, valine, and methionine were depleted when phenylalanine was elevated 20-fold(1, 2); but in human CSF leucine, isoleucine, and valine were unchanged, and methionine concentrations actually increased (Table I). Because the relationship between the concentrations of amino acids in CSF and brain is not known, it is not clear whether we are observing a major species difference in the response of rodent and human brain tissue to excessive phenylalanine or whether CSF simply provides a poor reflection of cerebral amino acids.

In the above study we observed few decreases in serum amino acid concentrations associated with high PA levels. This is contrary to previous reports(6,7). In fact, one author(8) has suggested that the low concentrations of serum amino acids may represent a shortage sufficient to deprive the brain of adequate metabolites for normal growth and differentiation and be causally linked with the subsequent mental retardation associated with phenylketonuria. Unfortunately, in these contradictory studies which demonstrated generalized decreases in serum amino acids, the absence of rigorous dietary control makes it impossible to ascertain what the critical metabolic variable or variables may be.

The comparison of subjects on unrestricted diets with those on synthetic diets low in phenylalanine introduces a number of dietary variables apart from the obvious difference in phenylalanine levels. This was evident in an earlier study of premature infants by Cockburn *et al*(9) in which the following 3 formulae were used sequentially in the same experimental subject:

1. Lofenalac® alone.
2. Lofenalac® + L-PA 100 mg/K/da.
3. Modified cow's milk (Enfamil®).

The two Lofenalac® formulae resulted in plasma concentrations of amino acids which were entirely comparable except for sporadically higher phenylalanine and tyrosine values using diet #2. On the other hand, the cow's milk formula produced significant alterations in plasma amino acid patterns. In this case, the qualitative difference in the diets was a more important variable than the presence or relative absence of a single essential amino acid. Much of the variability we observed in the amino acids of those phenylketonurics on unrestricted diets may be attributed to the qualitative and quantitative heterogeneity of their nutritional intake.

In addition, one final source of error must be avoided in gathering metabolic data during the initial period when the patient is unaccustomed to the synthetic diet and is reluctant to eat: For this period, which may last 2 weeks or more, there is invariably a considerable alteration in the amino acid patterns of blood, urine, and CSF.

Summary. Six phenylketonuric subjects were maintained on a rigorously controlled synthetic diet low in phenylalanine, to which phenylalanine was later added. The concentrations of amino acids in blood and CSF were compared under conditions of normal and high circulating phenylalanine levels. The concentrations of tyrosine, tryptophan, histidine, methionine, citrulline, glutamine, and the dipeptide, carnosine, in the CSF showed significant increases when the PA levels of phenylketonuric patients were permitted to rise 20-fold from normal levels. Only threonine and arginine decreased significantly. In serum the shift to high phenylalanine concentrations resulted in significant changes in only 3 of 18 amino acids. Threonine, histidine, and alanine were decreased. The discrepancy between these changes in serum amino acids and those reported by other investigators are best explained by differences in dietary control.

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Role of PMN-Leukocyte Lyosomes in Tissue Injury, Inflammation And Hypersensitivity. III. Passive Cutaneous Anaphylaxis Induced in The Rat with Homologous and Heterologous Hyperimmune Antibody.* (31307)

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The mechanism of passive cutaneous anaphylaxis (PCA) in the rat has long been a subject of controversy, particularly with regard to the possible roles of mediators such as histamine, serotonin and complement (C'). Thus Ovary(1) reported that an antihistamine, mepyramine, suppressed the reaction to heterologous precipitating antibody. Other investigators, however, showed that these reactions produced no mast cell damage(2). Furthermore, the response could not be inhibited by prior depletion of histamine and serotonin with compound 48/80 or by pretreatment with antihistamines and the serotonin antagonist BOL-148(3,4). Bier *et al* (5) and Osler and co-workers(6) reported a positive correlation between the intensity of the cutaneous reaction and the levels of circulating C'; yet Bloch *et al*(7) have shown that both non-C'-fixing guinea pig γ_1 and C'-fixing γ_2 antibody can sensitize the rat for PCA.

Polymorphonuclear (PMN) leukocytes had been observed in the lesions of PCA but were generally dismissed as unlikely to have a primary role in the reaction in either the guinea pig(8) or the rat(1). Brocklehurst and colleagues(3), however, had demonstrated that the amounts of heterologous antibody required to produce an Arthus reaction in the rat were not a great deal larger than those used to produce PCA and suggested that the two reactions might share a common pathogenetic mechanism. The Arthus reaction is mediated primarily by PMN-leukocytes, since the phenomenon cannot be elicited in leukopenic rabbits(9,10).

The recent description by Mota and others (11,12,13,14) of a heat-labile homologous, "mast cell-sensitizing" or "anaphylactic" antibody, capable of sensitizing the rat for PCA, has opened up new paths for investigation. In view of the marked differences between this antibody and hyperimmune antibody, as described by Binaghi *et al*(14), it seems possible that two more or less distinct mechanisms are involved in the production of the PCA lesion in the rat, the operative mechanism being dependent on the type of antibody used for sensitization. The present study investigates the PCA induced in the rat with hyperimmune antibody, either heterologous or homologous.

Materials and methods. Male Lewis rats (Microbiological Associates) were used throughout the study. Rats weighing 500-800 g were hyperimmunized with ferritin (Pentex, Kankakee, Ill.), using complete Freund's adjuvant. The ferritin had been dialyzed against several changes of phosphate buffered saline to remove cadmium. Four mg of antigen was given into the foot pads and intramuscularly in the gluteal region. The sera were collected at 4 weeks, pooled, and antibody nitrogen (AbN) was determined by the Kjeldahl method. Anti-ferritin was also

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