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Effect of Excess Phenylalanine and of Other Amino Acids on Brain Development in the Infant Rat

H. Peter Chase[34] and Donough O'Brien

B. F. Stolinsky Laboratories, Department of Pediatrics, University of Colorado Medical Center, Denver, Colorado, USA

Extract

The effects of excess phenylalanine and of a number of other amino acids on brain weight, total brain lipid, and formation of the myelin lipid cerebroside sulfate (sulfatide) have been studied in the newborn rat. Brain weight, amount of brain lipids, and the formation *in vivo* of sulfatide were reduced as a result of injections of phenylalanine or of certain other amino acids. Total brain cholesterol, cerebroside, and sulfatide were all significantly reduced in phenylalanine-injected animals, although sulfatide was the only lipid that was reduced per gram wet weight of brain. The *in vitro* activity of the brain enzyme, galactolipid sulfotransferase, which catalyzes the formation of sulfatide from cerebroside and adenosine 3′-phosphate 5′-phosphosulfate (PAPS), was significantly reduced only when exogenous PAPS was not added to the assay medium. This finding suggests that in the brains of animals injected with phenylalanine there was impairment in the formation of PAPS from ATP and sulfate. In both cerebrums and cerebellums of animals injected with phenylalanine for the first 18 days of life, the amount of brain DNA and protein was reduced.

Speculation

Increased ambient tissue levels of a number of amino acids, including phenylalanine, may interfere with normal development of the infant rat brain.

Introduction

The frequent association between mental retardation and errors of amino acid metabolism remains unexplained. One condition common to each of these syndromes may be an impairment of the formation of neuronal myelin. Histologically, this has been demonstrated in young patients with phenylketonuria and was associated with decreased brain weight [1]; nevertheless, quantitative measurements of the myelin lipids cerebroside and cerebroside sulfate (sulfatide) have shown conflicting results in this disease [7, 11, 17]. Decreased myelin has also been described in a patient dying of homocystinuria [13], and total brain lipids, especially cerebroside and sulfatide, have been reported to be decreased in patients with branched-chain ketoaciduria [20].

In the developing rat, the *in vivo* or *in vitro* synthesis of sulfatide has been shown to correlate with the period of rapid myelin formation [8, 16]. Sulfatide formation proceeds by two main steps: *1)* the formation of the active sulfate donor, PAPS, and *2)* the transfer of the sulfate to a mole of galactocerebroside.

(reaction 1)

$$SO_4^{2-} + 2\ ATP \longrightarrow PAPS + P \sim P + ADP$$

(reaction 2)

galactocerebroside + PAPS —(galactolipid sulfotransferase)→ sulfatide + PAP (phosphoadenosinephosphate)

The lipids cerebroside and sulfatide have been shown to be present in myelin in greater concentration than in other areas of the brain [18]. In addition, during the period of rapid growth of rat brain, the sulfatide formed has been shown to be located specifically in myelin [5]. In this study, the *in vivo* and *in vitro* formation of brain sulfatide, and the quantitative amounts of brain cerebroside, sulfatide, cholesterol, and total brain lipid have been studied in rat pups injected with phenylalanine. An attempt also has been made to determine or how long after birth phenylalanine must be administered to irreversibly decrease *in vivo* formation of brain sulfatide. In addition, the *in vivo* effects of several other amino acids were evaluated.

A reduction in the number of brain cells, as represented by brain DNA, or a reduction in the formation of brain protein, may also be important in causing the clinical picture produced by excess phenylalanine. The *in vitro* synthesis of protein by rat brain has previously been shown to be decreased in the presence of excess phenylalanine [2]. In this study, excess phenylalanine was given to rat pups for the first 18 days of life, and the cerebral and cerebellar protein and DNA contents were quantitated.

Materials and Methods

Rats of the Sprague-Dawley strain were injected subcutaneously within 12 h after birth with one of eight amino acids (5 g/kg/24 h), or phenylpyruvic acid (5 g/kg/24 h), or ammonium carbonate (2.5 g/kg/24 h). The dosage was divided into three daily portions and was continued for 18 days. Control animals were injected subcutaneously with 0.155 M NaCl. All pups were raised in nursing litters of 3 or 4 animals to 1 mother to provide similar nutrition, except for a group of 16 animals; this group was raised together in a large nursing litter to compare the effects of malnutrition with the effects of amino acid loading. Levels of amino acids in serum were assayed at various times following injection using a fluorescent technique for phenylalanine and tyrosine [19]; a high sensitivity modification of the automatic amino acid analyzer [25] was used for the remaining amino acids. The concentrations of amino acids were elevated 5–10 times the normal level in the first 4 h following injection, and returned to normal within 6 h. As injections were given every 8 h, high levels of amino acids were sustained throughout most of the day. Tyrosine levels also were elevated in animals injected with phenylalanine.

In initial experiments, the *in vivo* ^{35}S-sulfatide formation in myelin was determined in control animals and those injected with phenylalanine at different periods after birth. In later experiments, to determine the reversibility of the effects of excess phenylalanine, the injections of phenylalanine were discontinued at regular intervals and the animals allowed to nurse undisturbed until age 18 days, when the *in vivo* formation of sulfatide was determined. The formation of sulfatide in myelin was also determined in 18-day-old animals receiving one of seven amino acids, phenylpyruvic acid, or ammonium carbonate from the first day of life.

Determination of In Vivo *Sulfatide Synthesis*

In vivo synthesis of sulfatide was estimated in animals that received an intraperitoneal injection of ^{35}S-sulfate, 0.32 μCi/g body weight [8]. Twenty-four hours later the animals were decapitated and the brains were removed and weighed. The brain lipids were extracted with 19 volumes of chloroform–methanol, 2:1 (v/v) and partitioned by the method of FOLCH *et al.* [10]. The extracts were then dried and weighed. Dried lipids were dissolved in 10 ml of a scintillation fluid containing 2,5-diphenyloxazole (PPO), 5.0 g; 1,4-bis[-2-(5-phenyloxazolyl)]-benzene (POPOP), 0.3 g; methanol, 50 ml; and toluene, 950 ml. The radioactivity was then determined in a liquid scintillation counter [26].

Separation and Identification of Products

In a limited number of experiments (six control and six phenylalanine-injected animals, aged 12 and 18 days, respectively) 524 h following the ^{35}S-sulfate injection brain lipids were fractionated by Florisil [27] column chromatography [21]. Over 90% of the ^{35}S-sulfate incorporated into brain lipids was specifically incorporated into sulfatide.

In a similar number of experiments, myelin was purified by the method of EICHBERG *et al.* [9]. The brain homogenate was divided into six fractions: large myelin, small myelin, nuclei and mitochondria, synaptosomes, mitochondria, and tissue debris. During the period of active myelin formation, from 75 to 85% of ^{35}S-radioactivity incorporated into brain lipids was incorporated into the large and small myelin fractions in both the control animals and those injected with phenylalanine. This observation tended to verify the fact that sulfatide was primarily a 'myelin lipid' during the period under study.

Determination of In Vitro *Sulfatide Synthesis*

Three methods of assaying the activity were used: in method 1, ^{35}S-sulfate served as the ^{35}S-precursor;

in methods 2 and 3, ^{35}S-adenosine 3′-phosphate 5′-phosphosulfate (PAPS) served as the sulfatide precursor. Exogenous cerebroside was also added in method 2. Whole homogenate was used as the enzyme source for assay method 1, and an 18,000 × *g* supernatant was used for methods 2 and 3. Methods 1 and 2 were as described by MCKHANN [16], with the following modifications of the second assay: the brains used were homogenized in 2 volumes (w/v) of 0.05 M phosphate buffer, pH 7.4; they were then centrifuged at 18,000 × *g* for 20 min and the resulting supernatant used as a source of enzyme. The incubation media contained: (in μmoles) phosphate buffer, pH 7.4, 100; ethylenediaminetetraacetate (EDTA) 4; reduced glutathione, 10; imidazole, pH 7.4, 100; magnesium chloride, 10; potassium sulfate, 0.8; and 0.5 mg cerebroside [28] dissolved in 1% Brij 96 [29]. The third method of measuring galatolipid sulfotransferase was that described by BALASUBRAMANIAN [3] and by BACHHAWAT [personal communication, 1968]. In this method, endogenous brain cerebroside serves as the acceptor for the ^{35}S-PAPS; exogenous cerebroside is not included in the assay system. The incubation medium consisted of phosphate buffer, pH 7.4, 50 μmoles; EDTA, 25 μmoles; reduced glutathione, 5 μmoles; 0.1 ml of the 18,000 × *g* supernatant prepared as in method 2; and water to a final volume of 0.5 ml. After 1 h of incubation at 37° the reaction was stopped by adding 2.5 volumes of chloroform–methanol, 2:1. After separation of the two phases the protein pellet was removed and the lower phase was washed five times with 'theoretical upper phase' [8]. The lower phase was then dried and counted.

Quantitative Lipid Measurements

Brain lipids were extracted according to ROUSER *et al.* [22]. Cholesterol was determined by a modification of the Lieberman-Burchard reaction. Cerebrosides and sulfatides were initially separated from other brain lipids by Florisil column chromatography [21] and then were separated from one another by thin-layer chromatography using a 9:1 mixture of Silica Gel Plain [30] and magnesium silicate [31]. Plates were streaked with a known aliquot by weight of the cerebroside-sulfatide fraction from the Florisil column, and then were developed using the solvent system chloroform–acetone–methanol–glacial acetic acid–water, 5:2:1:1:0.5. Cerebroside and sulfatide bands were revealed by spraying with water and were removed individually from the plate with a razor blade. They were eluted from the adsorbent with chloroform–methanol–water, 2:1:0.1. The lipids were hydrolyzed and the hexose content determined by the orcinol reaction [23]. Factors of 4.55 and 5.08 were used to convert hexose values to cerebroside and sulfatide, respectively. Cerebroside and sulfatide standards run through the entire procedure showed recoveries of 102–104%.

DNA and Protein Measurements

DNA was measured by the method of ZAMENHOF *et al.* [24] and protein was measured by the technique of LOWRY *et al.* [15]. Brains were separated into cerebellum and cerebrum, with the latter referring to the sum of brain stem and cerebral cortex.

Results

Effects of Phenylalanine Loading

In the rat, sulfatide synthesis normally starts when the animal is 6 days old, reaches a peak at 18 days, and decreases to a low rate at 23 days (fig. 1). Animals injected with phenylalanine from day 1 of life began to synthesize sulfatide at the same time as did control animals, but at 10 days of age had a significantly lower sulfatide synthesis, and at 18 days of age the synthesis was 50% less than control animals. Body weights in experimental animals were not significantly depressed, although brain weights and total brain lipids were considerably lower than those found in control animals (table I). Lower concentrations of total brain cholester-

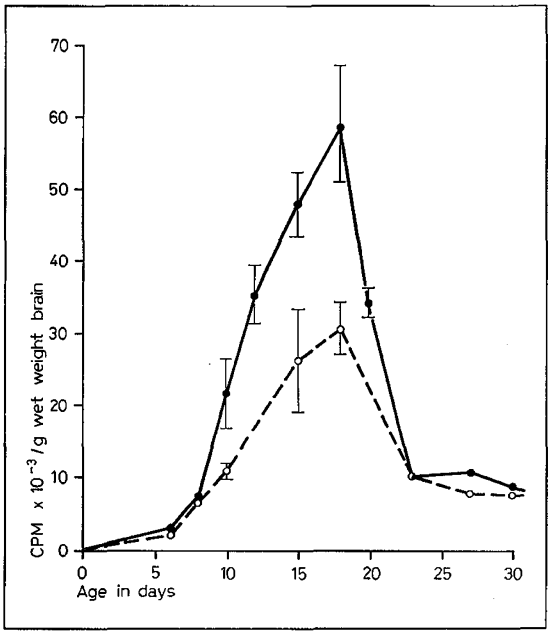

Fig. 1. The *in vivo* incorporation of ^{35}S-sulfate into rat brain sulfatide. The solid line represents control animals and the broken line animals injected with phenylalanine from birth until day of killing. Each point represents a minimum of four animals studied ±1 SD.

ol, cerebroside, and sulfatide were found in animals injected with phenylalanine, although these lipid levels were less obviously reduced when calculated as milligrams per gram wet weight of brain (table II). The quantity of brain sulfatide in animals injected with phenylalanine remained significantly lower ($P < 0.05$) when calculated per gram brain weight. Galactolipid sulfotransferase activity *in vitro*, using ^{35}S-sulfate as the sulfatide precursor, was significantly lower than in the control brains (table III). Activity was not significantly decreased in the presence of exogenous ^{35}S-PAPS. DNA and protein contents were decreased in both the cerebrums and the cerebellums of the animals injected with phenylalanine (table IV).

Reversibility of the Phenylalanine Effect

Phenylalanine loading in the first 4 days of life, followed by 14 days of normal nutrition, resulted in significantly reduced brain weight, total brain lipid weight, and synthesis of sulfatide *in vivo* in 18-day-old animals (table V). Effects were even more marked in 18-day-old animals receiving phenylalanine injections for the first 8 days of life. At a later age, 22 days, the decline in ^{35}S-sulfatide synthesis followed the pattern of control animals (fig. 1), with no evidence for a later increase in synthesis.

Effects of Other Amino Acids and Ammonium Carbonate

Of all the amino acids studied, methionine, injected

Table I. Effects of long-term amino acid loading in 18-day-old rats[1]

Exp. treatment	No. of animals	Body wt, g	Brain wt, g	Total brain lipid, mg	^{35}S-sulfate incorporation, cpm/g wet brain
Controls	24	46.2± 4.9[2]	1.403±0.061	75.4±3.6	58,967±8,421
Poorly nourished	18	21.4± 4.8	1.231±0.074	61.8±5.1	29,880±6,259
Phenylalanine	11	40.0± 4.5	1.141±0.084	56.6±5.2	30,656±3,497
Phenylpyruvate	8	36.3± 6.6	1.297±0.057	70.5±4.7	57,604±6,397
Isoleucine	8	44.0± 3.6	1.282±0.058	72.8±4.7	53,869±3,810
Histidine	8	39.1± 8.8	1.156±0.086	60.6±3.9	39,254±8,017
Tyrosine	8	35.0± 3.3	1.210±0.037	60.9±3.8	28,110±8,499
Methionine	4	33.0± 2.5	1.166±0.046	60.1±0.9	5,773±2,811
Arginine	7	49.4± 4.2	1.313±0.046	70.1±1.6	22,561±6,029
Leucine	8	35.8± 2.2	1.286±0.029	66.9±1.3	43,961±8,294
Valine	7	42.0±11.1	1.294±0.055	62.8±4.3	42,190±6,590
$(NH_4)_2CO_3$	6	40.2± 4.8	1.241±0.029	66.4±2.1	37,572±5,671

[1] In all categories, the figures for the treated animals were significantly lower ($P < 0.01$) than the controls, using Student's t test, except as follows: body weights were not significantly different for isoleucine- and arginine-loaded animals, and only marginally significant ($P < 0.05$) for histidine-loaded animals. Total brain weights and ^{35}S-sulfate incorporation were not significantly different for phenylpyruvate- and isoleucine-loaded animals or for arginine-loaded animals (brain weight only).

[2] Values represent the mean ±1 SD.

Table II. Cholesterol, cerebroside, and sulfatide in 18-day-old rats

Exp. treatment	Cholesterol		Cerebroside		Sulfatide	
	Total per brain, mg	mg/g wet wt brain	Total per brain, mg	mg/g wet wt brain	Total per brain, mg	mg/g wet wt brain
Phenylalanine-injected animals	9.2±1.0	8.7±2.5	1.492±0.451	1.396±0.688	0.535±0.209	0.504±0.101
Control animals	12.6±0.7	9.0±0.6	2.206±0.873	1.563±0.983	1.036±0.124	0.732±0.114
P value	< 0.01	> 0.10	> 0.1	> 0.1	< 0.02	< 0.05

[1] The control and test groups consisted of four animals each.

[2] Values represent mean ±1 SD; statistics were determined as in table I.

Table III. In vitro galactolipid sulfotransferase activity[1] in 18-day-old rats

Exp. treatment	^{35}S-sulfatide precursor		
	^{35}S-sulfate (assay 1)	^{35}S-PAPS plus exogenous cerebroside (assay 2)	^{35}S-PAPS (assay 3)
Control animals[2]	130± 7[3]	937±103	2,317±606
Phenylalanine-injected animals[2]	92±16	795±142	1,595±457
P value	<0.05	>0.5	>0.5

[1] Activity is expressed in cpm/mg protein.
[2] Control and test groups consisted of four or six animals each.
[3] Values represent mean ± 1 SD; statistics were determined as in table I.

subcutaneously (5 g/kg/24 h), inhibited formation of sulfatide *in vivo* to the greatest degree (table I) and was also associated with the greatest mortality rate (50%). Injections of phenylalanine, tyrosine, and arginine all inhibited sulfatide formation about 50% at 18 days of age; histidine, leucine, and valine did not inhibit myelin lipid formation to this extent; and injections of isoleucine had no inhibitory effects. The toxicity of the amino acids could have resulted from ammonia toxicity; to investigate this possibility a group of animals received ammonium carbonate; sulfatide formation was inhibited 37% by ammonium carbonate, and total brain weights and brain lipid weights were also decreased (table I).

Discussion

In these experiments, phenylalanine loading of newborn rats resulted in a reduction in brain weight and lipid content. Cerebroside and sulfatide, the predomi-

Table IV. DNA and protein in 18-day-old rats

Exp. treatment	Total DNA, mg		Total protein, mg		Ratio of brain wt, g/DNA, mg	
	Cerebrum	Cerebellum	Cerebrum	Cerebellum	Cerebrum	Cerebellum
Phenylalanine-injected animals[1]	0.846±0.042[2]	1.057±0.091	75.33±8.64	14.25±5.80	1.13	0.128
Control animals[1]	1.023±0.032	1.498±0.112	104.61±2.54	19.18±4.72	1.19	0.120
P value	<0.001	<0.001	<0.001	>0.5	>0.5	>0.5

[1] The control and test groups consisted of six animals each.
[2] Values represent mean ±1 SD; statistics were determined as in table I.

Table V. Effects of phenylalanine loading in 18-day-old rats

Days on L-phenylalanine	No. of animals	Body wt, g	Brain wt, g	Total brain lipid, mg	^{35}S-sulfate incorporation, cpm/g wet brain[1]
0 (controls)	8	43.0±0.03[2]	1.43±0.03	76.8±2.4	57,885±6,740
4	8	48.1±1.4	1.28±0.03	72.5±2.2	47,242±6,977
8	4	47.5±1.2	1.22±0.04	63.8±2.3	44,930±5,398
10	4	44.4±4.2	1.27±0.05	69.7±3.7	23,039±4,800
12	8	39.5±7.7	1.15±0.13	58.5±8.7	20,000±4,129
18	11	40.0±4.5	1.14±0.08	56.6±5.2	30,656±3,497

[1] The effect of phenylalanine is significant at the $P < 0.01$ levels for brain weight, total brain lipid, and ^{35}S-sulfate incorporation for all periods of administration. The slightly higher rate of ^{35}S-sulfate incorporation at age 18 days reflects a summary of animals studied at various intervals during the entire period of investigation; statistics were determined as in table I.
[2] Values represent the mean ±1 SD.

nant myelin lipids, were also significantly decreased, although only sulfatide was decreased in proportion to wet weight. Cholesterol, which appears in similar concentrations in all brain membranes, was less strikingly affected.

Sulfatide is formed as a result of two reactions: *1)* the synthesis of the active sulfate donor, PAPS, and *2)* the transfer of a sulfate molecule from PAPS to cerebroside in the presence of galactolipid sulfotransferase *(reactions 1* and *2)*. In the *in vivo* experiments, the onset and peak periods of sulfatide-forming activity were similar in animals treated with phenylalanine and in control groups (fig. 1), although sulfatide formation in the treated animals was uniformly lower. The lower quantitative levels of sulfatide in phenylalanine-injected animals confirm the experiments showing decreased *in vivo* formation. The *in vitro* assays of galactolipid sulfotransferase activity suggest a mechanism for the decreased sulfatide formation. When ^{35}S-sulfate was used as the precursor, sulfatide formation was significantly reduced; however, when exogenous PAPS was provided as the sulfate donor, in the presence or absence of exogenous cerebroside, there was no significant difference in activity. The inference, therefore, is that the effect of phenylalanine is to cause a reduction in the availability of PAPS, possibly as a result of decreased ATP. It is of interest that ammonium carbonate also decreased sulfatide formation, since ammonium carbonate also decreased sulfatide formation, since ammonium acetate has been shown to decrease concentrations of α-ketoglutaric acid in mouse brain [4], an intermediate in the ATP-producing citric acid and γ-aminobutyric acid cycles.

In studies in which phenylalanine injections were discontinued at various ages after birth, the period just prior to the onset of rapid sulfatide formation (the first 8 days of life) was found to be critical to normal brain development. The period of human brain development, comparable to the first 8 days of life in the rat, would occur prior to birth. Evidence already indicates that, in the human, elevated intrauterine levels of phenylalanine lead to permanent brain damage, even when these levels return to normal after delivery [12].

The decreased amounts of DNA and protein found in the cerebrums and cerebellums of phenylalanine-injected animals suggest that the toxicity of phenylalanine is broad and is not limited only to a reduced formation of myelin lipids. DNA, as a measurement of the number of cells of an organ, is reliable as long as the amount of DNA is constant per cell through the organ. Thus, DNA can probably be used as an estimate of the number of cells in the cerebrum, but polyploidy, with varying amounts of DNA per cell, is known to exist in the cerebellum [14]. DNA was significantly reduced in both cerebrum and cerebellum in 18-day-old animals injected from birth with phenylalanine. The effect was slightly greater in the cerebellum, with a mean reduction of 29%, compared with 17% in the cerebrum. Since formation of cerebral DNA in the rat is normally completed by 19 days of age, it might be anticipated that in animals loaded for 18 days there would be no further increase in cell number with age.

The reduction in DNA was of particular interest in relation to undernutrition. This condition also reduces myelin lipid formation [5], but causes little or no decrease in cerebral DNA. The main effect under these circumstances is on the cerebellum [6]. In addition, body weights were only slightly reduced in animals injected with phenylalanine compared with control animals, yet the brain weights and total lipid weights were reduced more in phenylalanine-injected animals than in undernourished animals of less than half the body weight (table I).

Other amino acids decreased brain weight and inhibited myelin lipid formation. Methionine, levels of which are usually elevated in blood of patients with homocystinuria, was associated with the greatest reduction in sulfatide formation. Methionine is a sulfur-containing amino acid, however, and changes in the intracerebral sulfate pools following methionine injections cannot be ruled out. Of the three amino acids known to be present in elevated amounts in blood of patients with maple syrup urine disease, leucine and valine slightly inhibited myelin lipid formation, while isoleucine caused no decrease. Arginine, a growth hormone-releasing factor, was associated with a slight increase in rat body weight and an increase in rat skeletal maturation, as determined by x-ray. Sulfatide formation, however, was decreased greater than 50% in arginine-injected animals. Histidine, elevated in the human disease histidinemia, also reduced brain weight and sulfatide formation in the rat.

Summary

The formation of brain sulfatide in infant rats *in vivo* was markedly inhibited by phenylalanine, methionine, tyrosine, arginine, and, to a lesser extent, by leucine and valine. Quantitative levels of the myelin lipids, cholesterol, cerebroside, and sulfatide were reduced in 18-day-old rats injected with phenylalanine from day 1 of life. The activity of the sulfatide-forming enzyme, galactocerebroside sulfotransferase, *in vitro* was reduced only in the absence of exogenous PAPS, suggesting that the mechanism of decreased sulfatide formation is related to diminished availability of PAPS. Total brain DNA and protein were also reduced in the phenylalanine-injected animals.

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34. Request for reprints should be addressed to: H.PETER CHASE, M.D., B.F.Stolinsky Laboratories, Department of Pediatrics, University of Colorado Medical Center, 4200 East Ninth Avenue, Denver, CO 80220 (USA).

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