Effect of Phenylalanine on Protein Synthesis in the Developing Rat Brain

By H. C. AGRAWAL, A. H. BONE AND A. N. DAVISON

*Medical Research Council, Membrane Biology Group, Department of Biochemistry,
Charing Cross Hospital Medical School, London W.C.2, U.K.*

(Received 17 November 1969)

1. Inhibition of the rate of incorporation of [^{35}S]methionine into protein by phenylalanine was more effective in 18-day-old than in 8-day-old or adult rat brain. 2. Among the subcellular fractions incorporation of [^{35}S]methionine into myelin proteins was most inhibited in 18-day-old rat brain. 3. Transport of [^{35}S]methionine and [^{14}C]leucine into the brain acid-soluble pool was significantly decreased in 18-day-old rats by phenylalanine (2mg/g body wt.). The decrease of the two amino acids in the acid-soluble pool equalled the inhibition of their rate of incorporation into the protein. 4. Under identical conditions, entry of [^{14}C]glycine into the brain acid-soluble pool and incorporation into protein and uptake of [^{14}C]acetate into lipid was not affected by phenylalanine. 5. It is proposed that decreased myelin synthesis seen in hyperphenylalaninaemia or phenylketonuria may be due to alteration of the free amino acid pool in the brain during the vulnerable period of brain development. Amyelination may be one of many causes of mental retardation seen in phenylketonuria.

In phenylketonuria most investigators mention incomplete myelination or possibly demyelination of the central nervous system as a prominent pathological feature (Alvord, Stevenson, Vogel & Engle, 1950; Poser & van Bogaert, 1959; Malamud, 1966). Analysis of the affected brains in this disease shows significant decreases in the concentration of such typical myelin constituents as cerebroside, cholesterol and proteolipids (Crome, Tymm & Woolf, 1962; Menkes, 1966, 1967; Prensky, Carr & Moser, 1968). The question whether or not these pathological and biochemical changes are manifestations of decreased myelin synthesis still remains unanswered. Menkes (1968) has suggested that in phenylketonuria deficiency in myelin lipid content may be the result of primary impairment in myelin protein synthesis. However, there is little experimental information obtained with intact animals to support this hypothesis. Inhibition of ribosomal protein synthesis by phenylalanine has been reported in relatively immature rabbit brain (Swaiman, Hosfield & Lemieux, 1968). In acute experiments with very high concentrations of phenylalanine, synthesis of brain cerebroside and sterol has been shown to be inhibited in developing rabbit brain (Barbato, Barbato & Hamanaka, 1968). The question whether high concentrations of phenylalanine alter the synthesis of a specific lipid during the period of rapid myelination was also investigated by Shah, Peterson & McKean (1968). Incorporation of [^{14}C]galactose into cerebral

glycolipids has also been shown to be inhibited in hyperphenylalaninaemia (Shah & McKean, 1968). To determine whether or not phenylalanine exerts its toxic effect on both myelin protein and lipid synthesis or if its effect is specific to one component only, we have examined the effect of hyperphenylalaninaemia in rats on the incorporation of labelled amino acids and acetate into brain proteins and lipids. Preliminary accounts of this work have been reported (Agrawal, Bone & Davison, 1969a,b).

MATERIALS AND METHODS

Chemicals. A.R. chemicals were used without further purification. Deionized water was used for making up all reagents. Crystalline bovine serum albumin (fraction V) and L-phenylalanine were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. AnalaR 98% (w/v) formic acid was purchased from British Drug Houses Ltd., Poole, Dorset, U.K. L-[^{35}S]Methionine (188mCi/mmol), L-[U- ^{14}C]leucine (6.5mCi/mmol), [U- ^{14}C]glycine (11.3mCi/mmol) and [1- ^{14}C]acetate (40mCi/mmol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. L-Phenylalanine (20%, w/v) suspension in 0.9% NaCl solution containing 2% (w/v) of Tween 80 was prepared by homogenizing L-phenylalanine in an Ultra-Turrax homogenizer (Scientific Instrument Centre, London W.C.1, U.K.) for 2 min and filtering through cheesecloth.

Protein determination. Protein insoluble in trichloroacetic acid was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951) and proteolipid protein by a modification of the same method described by Hess &

Lewin (1965). Crystalline bovine serum albumin was used as standard.

Animals. Laboratory-bred albino rats of known age from litters decreased in size to six to eight at birth were used throughout this study. Young adult rats (100–150g) of both sexes were used. All animals received standard diet. To minimize the effects of starvation and hypothermia the young rats were returned to the mother immediately after injections. Young (18-day-old) and adult rats were injected intraperitoneally with 2.0mg of phenylalanine/g body wt. as a 20% (w/v) suspension and the litter-mate controls received an equal volume of 0.9% NaCl containing 2% (w/v) of Tween 80. Radioactive precursors dissolved in 0.9% NaCl solution were injected into the animals (0.1 μ Ci/g body wt.) intraperitoneally 1 h after phenylalanine administration. Animals were killed 3 h after the administration of labelled compound. Eight-day-old animals were given both phenylalanine and radioactive compounds subcutaneously. The animals were killed by decapitation and the brains removed as quickly as possible, transected at the level of pons-medulla, washed with 0.9% NaCl solution, dried with filter paper and weighed in cooled glass tubes.

Blood samples. Immediately after decapitation of the rats, blood was collected in chilled centrifuge tubes and allowed to clot. Serum was separated by centrifugation in a clinical centrifuge. Serum phenylalanine was determined fluorimetrically by the method of McCaman & Robins (1962).

Procedure for the isolation of proteolipid protein. Brains were homogenized in 19vol. of chloroform-methanol (2:1, v/v) for 1 min in an Ultra-Turrax homogenizer, and the extract was filtered through a sintered-glass filter. The extract was partitioned against 0.2vol. of water as described by Folch, Lees & Sloane-Stanley (1957). The two phases were left to separate in a refrigerator overnight. The upper phase was carefully siphoned off by suction and the lower phase washed three times with equal volumes of synthetic upper phase (chloroform-methanol-water, 3:47:48, by vol.) and the lower phase was finally made to one phase with methanol. The upper phase and washings were discarded. Portions (10ml) of chloroform-methanol-soluble extract were made biphasic in a scintillation vial by adding a minimum quantity of water. The mixture was then evaporated to dryness under a stream of air. The residue, containing lipid and proteolipid protein, was washed successively three times with 5% (w/v) trichloroacetic acid and finally with ethanol-ether (1:1, v/v), centrifuged and the lipid-containing supernatant discarded. The vials were dried in a vacuum desiccator over silica gel. After this drying 0.2ml of 98% (w/w) formic acid was added to dissolve the protein followed by 10ml of scintillation fluid.

Treatment of chloroform-methanol-insoluble residue. The residue was washed repeatedly on a sintered-glass disc with 5% (w/v) trichloroacetic acid until it was free of acid-soluble radioactive compounds. The acid-extracted residue was treated five times with portions (10ml) of ethanol-ether (1:1, v/v) to remove trichloroacetic acid and lipid material. The pellet was dried in a vacuum desiccator over silica gel. The dried pellet was suspended in 5ml of 98% (w/w) formic acid, shaken vigorously in a vortex mixer and usually left overnight in the refrigerator to render the protein completely soluble. Portions of the

solution (equivalent to 1.0mg of protein) were transferred to scintillation vials, followed by 10ml of scintillation fluid. Protein was determined after evaporating the formic acid *in vacuo* over NaOH.

Isolation of free amino acids and acid-insoluble protein. Brains were frozen on solid CO₂, weighed and homogenized in ice-cold 6% (w/v) HClO₄ (5ml/g of tissue) in an Ultra-Turrax homogenizer for 1 min at 0°C. The homogenate was centrifuged at 20000g for 30 min in a MSE 50 ultracentrifuge. The clear supernatant was carefully poured into an ice-cold beaker and the residue washed twice with portions (2ml) of 6% (w/v) HClO₄ and centrifuged. The washings were combined with the original extract. The supernatant solution was adjusted to pH 4.0 with cold 2M-KOH and after the suspension had been left overnight at 0–4°C the precipitated KClO₄ was separated by centrifugation at 20000g for 30 min. The residue was washed with ice-cold water. The combined extract was filtered through Whatman no. 54 filter paper and made up to a known volume (25ml/g of tissue) with ice-cold water. Suitable portions of the clear fluid were analysed for free amino acids by using a Technicon amino acid analyser. Another portion of the extract was evaporated to dryness under reduced pressure. The dried residue was taken up in 98% w/w formic acid (4ml/g of tissue), mixed thoroughly, and centrifuged, and the clear supernatant was used for liquid-scintillation counting.

The perchloric acid-insoluble residue was heated with 5% (w/v) trichloroacetic acid containing 0.5% (w/v) of the appropriate unlabelled amino acid at 90°C for 15 min to extract nucleic acids, washed three times with 10ml of ethanol-ether (1:1, v/v) at room temperature and dried *in vacuo*. The dried protein was suspended by vigorous shaking in 5ml of 98% (w/w) formic acid and went into solution after storage overnight at 0–4°C. Portions of the clear solutions were used for protein and radioactivity determinations.

Determination of radioactivity. The radioactivity of ¹⁴C and ³⁵S-labelled samples was measured in a Beckman scintillation spectrometer essentially by the technique of Hall & Cocking (1965) at an efficiency of 70%. Quenching was determined both by the channels-ratio method and by the use of an internal standard. The composition of the scintillation fluid was 4g of 2,5-diphenyloxazole, 0.1g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 700ml of toluene and 300ml of ethanol. The samples were transferred to the scintillation spectrometer and were left for 2 h before measurement of their radioactivity. The radioactivity of each sample was counted once for 50 min, to an error of $\pm 0.7\%$.

Subcellular fractionation. Myelin was prepared from brains of 18-day-old rats essentially by the technique of Autilio, Norton & Terry (1964). Briefly, about 4g of tissue was subjected to mild homogenization in 0.32M-sucrose with a motor-driven Teflon-glass homogenizer with four up-and-down strokes for 90s. The homogenate was diluted to a final concentration of 5% (w/v) with 0.32M-sucrose. Portions of the homogenate (equivalent to 700mg wet wt. of tissue) were layered over 0.85M-sucrose and centrifuged in a swing-out rotor (no. 2418; MSE 50 centrifuge) at 75000g for 30 min. Myelin was collected at the interface, subjected to osmotic shock with 9 vol. of ice-cold distilled water, left overnight at 0°C and then centrifuged at 12000g to obtain purified myelin.

The pellet was washed twice with 0.32M-sucrose. Proteolipid protein from the fractions was isolated immediately by the procedure of Folch *et al.* (1957).

RESULTS

Before investigation of the effect of injected phenylalanine on the rate of incorporation of radioactive amino acids into the brain protein of 18-day-old rats an attempt was made to maintain serum phenylalanine concentrations for a period of 4 h at a value close to those found in phenylketonuric patients (Fig. 1). Although after a single injection of phenylalanine there was a considerable fluctuation, the mean serum phenylalanine concentration

Fig. 1. Serum phenylalanine concentration after injection of L-phenylalanine into 18-day-old rats. Phenylalanine was given (2mg/g body wt.) by intraperitoneal injection and blood collected at intervals for phenylalanine determination (see the Materials and Methods section). Serum phenylalanine concentration in treated animals (■) is compared with that of control rats (○).

4 h after phenylalanine administration in 18-day-old rats was 57 ± 23 mg/100ml. The saline-injected animals had a mean serum phenylalanine concentration of 2.0 ± 0.1 mg/100ml. The concentration of phenylalanine in the acid-soluble extract of brain from 18-day-old rats 4 h after injection of phenylalanine (2mg/g) was found to be $0.85 \mu\text{mol/g}$ wet wt. of tissue. The concentration in untreated rat brain of the same age is $0.05 \mu\text{mol}$ of phenylalanine/g wet wt. of tissue (Agrawal, Davis & Himwich, 1966).

Influence of L-phenylalanine on the rate of incorporation of [^{35}S]methionine and [^{14}C]leucine into chloroform-methanol-soluble and -insoluble protein of developing rat brain. Because both [^{35}S]methionine and [^{14}C]leucine are rapidly converted into other amino acids (Gaitonde & Richter, 1956; Berl & Frigyesi, 1967) the specific radioactivity of leucine and methionine was not determined in either the acid-soluble pool or the protein fractions. Results are recorded as incorporation of ^{35}S - and ^{14}C -labelled amino acids into the acid-soluble pool and protein of brain. The inhibition in the rate of incorporation of ^{35}S -labelled amino acids into the proteolipid and other brain protein in hyperphenylalaninaemic rats was found to be greater in 18-day-old animals than in immature (8-day-old) and young adult rats (Table 1). Therefore further studies were carried out on 18-day-old rats. Phenylalanine blocked the incorporation of ^{35}S -labelled amino acids into the chloroform-methanol-soluble (proteolipid protein) and -insoluble protein by 75% and 69% respectively of brain from 18-day-old rats (Table 1). When these experiments were repeated with [^{14}C]leucine as precursor uptake of ^{14}C -labelled amino acids

Table 1. *Influence of phenylalanine on incorporation of [^{35}S]methionine into the chloroform-methanol-soluble and -insoluble protein fractions of developing and adult rat brains*

Phenylalanine (2.0 mg/g body wt.) was injected 1 h before injection of [^{35}S]methionine ($0.1 \mu\text{Ci/g}$ body wt.). After 3 h the rats were killed and protein extracts prepared as described in the Materials and Methods section. Serum phenylalanine concentration and radioactivity in protein fractions were determined as described in the Materials and Methods section. Mean values $\pm$ S.D. are recorded with the numbers of animals examined in parentheses. *P* values were determined by Student's *t* test.

Age (days)		Concn. of serum phenylalanine (mg/100 ml)	Chloroform-methanol-soluble protein (d.p.m./mg of protein)	Chloroform-methanol-insoluble protein (d.p.m./mg of protein)
8	Control	4.6 ± 2.6 (5)	708 ± 123 (4)	2058 ± 72 (4)
	Experimental	53.0 ± 34 (7)	320 ± 60 (4)	1181 ± 115 (4)
	<i>P</i> value		<0.005	<0.001
18	Control	3.0 ± 0.5 (6)	682 ± 159 (6)	882 ± 238 (6)
	Experimental	51.0 ± 20 (6)	171 ± 31 (6)	274 ± 44 (6)
	<i>P</i> value		<0.001	<0.001
Adult	Control	2.0 ± 0.1 (3)	222 ± 9.0 (3)	749 ± 56 (3)
	Experimental	13.0 ± 4.0 (3)	103 ± 8 (3)	442 ± 71 (3)
	<i>P</i> value		<0.001	<0.005

Table 2. Influence of phenylalanine on the incorporation of labelled leucine and acetate into brain proteins and lipid

The 18-day-old rats were injected with phenylalanine (2.0 mg/g body wt.) and 1 h later with [U-¹⁴C]leucine or [1-¹⁴C]acetate. Animals were killed 3 h after injection of radioactive precursor and brain fractions isolated and analysed as described in the Materials and Methods section. Serum phenylalanine concentrations were 2.0 ± 0.1 (18) for control rats and 57 ± 23 (18) mg of phenylalanine/100 ml of serum for the phenylalanine-treated animals. The numbers of animals used in each group are shown in parentheses and mean radioactivities $\pm$ s.d. are recorded. *P* values were determined by Student's *t* test; N.S., not significant.

Precursor ...	[U- ¹⁴ C]Leucine		[1- ¹⁴ C]Acetate
	Proteolipid protein (d.p.m./mg of protein)	Chloroform-methanol-insoluble protein (d.m.p./mg of protein)	Lipid (d.p.m./mg wet wt. of tissue)
Control	517 ± 178 (7)	1003 ± 175 (7)	43 ± 0.1 (5)
Experimental	272 ± 87 (6)	549 ± 175 (7)	47 ± 2 (5)
<i>P</i> value	<0.01	<0.001	N.S.

into the proteolipid and chloroform-methanol-insoluble protein was 48% and 45% respectively (Table 2).

To determine whether or not phenylalanine has a general toxic effect on both protein and lipid biosynthesis, or whether its action is more specific to one component, incorporation of [1-¹⁴C]acetate into the total brain lipid was examined. Under identical experimental conditions incorporation of acetate into brain lipid was not affected (Table 2). These observations suggest that *in vivo* the elevated circulating concentrations of phenylalanine block incorporation of amino acids into protein and that inhibition of lipid synthesis in chronic conditions may therefore be a secondary manifestation of the primary impairment of protein metabolism (see below).

Myelin protein synthesis in hyperphenylalaninaemia. It was considered that phenylalanine might exert its toxic effect either selectively on myelin proteins or through a general inhibitory action on the proteins in all subcellular particles. To ascertain which of these alternatives was most likely, myelin was isolated by the procedure given in the Materials and Methods section. It was found that phenylalanine decreased the rate of incorporation of ³⁵S-labelled amino acids into myelin protein *in vivo*. The degree of inhibition for both proteolipid and the trichloroacetic acid-insoluble proteins of myelin was higher than for other subcellular fractions (Fig. 2). Since the inhibition of incorporation into myelin protein was relatively greater than that for other subcellular fractions and since these are contaminated to some extent with myelin fragments it is possible that hyperphenylalaninaemia during the active period of myelination may have a deleterious and irreversible effect primarily on the formation of myelin proteins.

Effect of increasing dose of phenylalanine on

Fig. 2. Inhibition of incorporation of [³⁵S]methionine into the proteins of subcellular fractions and myelin of brain from 18-day-old rats. Experimental conditions were as described in the Materials and Methods section. Incorporation into the proteolipid (□) and total protein (■) is shown. Protein was prepared from myelin and the combined subcellular fractions obtained after removing myelin by the method of Autilio *et al.* (1964). In this experiment incorporation into total (trichloroacetic acid-insoluble) protein was measured as only a very small chloroform-methanol-insoluble residue can be obtained from myelin. Results are the means of two separate experiments on six rats.

incorporation of amino acids into protein. A progressive decrease was observed in the rate of incorporation of ³⁵S-labelled amino acids both into proteolipid and insoluble protein with increasing dose of phenylalanine (Fig. 3), but the elevated serum phenylalanine concentrations in this experi-

ment had no direct relationship with decreased incorporation of ^{35}S -labelled amino acids into the brain protein. These results must be regarded as preliminary, since they were obtained from only a limited number of experiments.

Fig. 3. Effect of different doses of phenylalanine on incorporation of ^{35}S methionine into the brain protein fractions of developing rat brain. The 18-day-old rats were given intraperitoneal injections of phenylalanine [20% (w/v) in 0.9% NaCl solution containing 2% (w/v) of Tween 80 in relation to body wt.]. Then 1 h afterwards animals were given intraperitoneal injections of ^{35}S methionine (0.1 $\mu\text{Ci/g}$ body wt.). At 3 h later animals were killed and brain proteolipid (□) and chloroform-methanol-insoluble protein (■) extracted. Samples were prepared and radioactivity was determined as described in the Materials and Methods section. Each bar represents the results for one experiment in which samples from three brains were pooled; six separate determinations were made for the group given 2 mg of L-phenylalanine/g body wt.

Inhibition of transport of amino acids in brain in hyperphenylalaninaemia. In order to understand the mechanism by which high concentrations of serum phenylalanine *in vivo* alter the rate of incorporation of ^{35}S methionine and ^{14}C leucine into protein the concentrations of ^{35}S -labelled and ^{14}C -labelled amino acids of the brain acid-soluble pool were measured. It was found that transport of ^{35}S methionine and ^{14}C leucine into the brain acid-soluble pool of phenylalanine-treated animals was decreased to 65% and 40% respectively. The decrease in the two amino acids in the acid-soluble pool equalled the decrease in the rate of incorporation of ^{35}S methionine and ^{14}C leucine into the protein (Table 3). On the other hand, ^{14}C glycine entry into the acid-soluble pool was not affected by phenylalanine under the same experimental conditions. These observations suggest that in acute experiments the elevated phenylalanine concentration interferes with the transport of only certain amino acids into neural tissue *in vivo*. The diminished uptake of radioactive amino acids into the acid-soluble pool might explain the inhibition in the rate of incorporation of amino acids into protein.

DISCUSSION

There is ample evidence in human phenylketonuria that there is a deficiency in hepatic phenylalanine hydroxylase activity (Jervis, 1953; Wallace, Moldave & Meister, 1957; Mitoma, Auld & Udenfriend, 1957; Kaufman, 1958). However, it remains to be established how the resulting elevated concentrations of plasma and intracellular phenylalanine or its metabolites adversely affect brain development. Faulty myelination has been clearly established in about one-third of all cases examined histologically and, as there is no evidence of active myelin destruction in the disease, the deficiency in

Table 3. Effect of phenylalanine loading on the entry and incorporation of labelled amino acids into the developing rat brain

The 18-day-old rats were given phenylalanine (2 mg/g body wt.) by intraperitoneal injection. Radioactive amino acids (0.1 $\mu\text{Ci/g}$ body wt.) were injected 1 h later and 3 h after this the animals were killed. Experimental procedure was exactly as described in Table 2. Preparation of perchloric acid extracts and technique for radioactivity determination are given in the Materials and Methods section. Results are the means $\pm$ S.D. together with the numbers of observations in parentheses. *P* values were determined by Student's *t* test; N.S., not significant.

Precursor ...	^{35}S Methionine		^{14}C Leucine		^{14}C Glycine	
	Acid-soluble (d.p.m./g of tissue)	Protein (d.p.m./mg of protein)	Acid-soluble (d.p.m./g of tissue)	Protein (d.p.m./mg of protein)	Acid-soluble (d.p.m./g of tissue)	Protein (d.p.m./mg of protein)
Control	14102 $\pm$ 613 (4)	939 $\pm$ 194 (4)	9591 $\pm$ 1777 (4)	13381 $\pm$ 2781 (4)	8059 $\pm$ 2566 (6)	192 $\pm$ 35 (6)
Experimental	4972 $\pm$ 617 (4)	360 $\pm$ 97 (4)	5833 $\pm$ 2366 (4)	6839 $\pm$ 3111 (4)	9975 $\pm$ 2760 (6)	161 $\pm$ 46 (6)
Inhibition (%)	65	62	40	48	—	16
<i>P</i> value	<0.005	<0.001	<0.05	<0.02	N.S.	N.S.

myelin is probably due to inadequate synthesis rather than to demyelination (Poser & van Bogaert, 1959; Woolf, 1963). Menkes (1968) proposed that myelin protein synthesis is impaired in phenylketonuria. Barbato *et al.* (1968), investigating the incorporation of labelled precursors into brain tissue of 15–20-day-old rabbits *in vivo*, found no inhibition of incorporation of tritiated cytidine into RNA of the microsomes, mitochondria or nuclei, but significant inhibition of incorporation of [^{14}C]acetate into myelin lipids. The concentrations of serum phenylalanine required for this to occur were very high and with concentrations of the same order as those used in our own experiments no inhibition was observed (see Tables 1 and 2). In the present work the dose of phenylalanine was chosen in order to maintain the serum phenylalanine concentration for a period of 4h close to those found in severe cases of phenylketonuria. Concentrations of up to 79mg of phenylalanine/100ml of serum are found in untreated phenylketonuric children (Hackney, Hanley, Davidson & Linsao, 1968). The short period of the experiment eliminated the possibility of changes in nutritional status, body weight and the high mortality rate seen in many chronic feeding regimes. It was found in our experiments that incorporation of [$1\text{-}^{14}\text{C}$]acetate into brain lipid was not significantly inhibited in hyperphenylalaninaemia. It therefore seemed reasonable to suggest that in chronic conditions of hyperphenylalaninaemia decrease in lipid synthesis may be secondary to an impairment of myelin protein synthesis.

Phenylalanine as an inhibitor of transport of amino acids into the developing rat brain. Our evidence suggests that elevated serum phenylalanine concentrations inhibit the cerebral uptake of free amino acids, thus lowering the supply of amino acids needed for protein biosynthesis in developing brain. The distorted pattern of free amino acids in brain in hyperphenylalaninaemia has been observed by various investigators. The alterations in the free amino acid pool are, however, apparently neither a consistent nor a reproducible finding (Carver, 1965; McKean, Boggs & Peterson, 1968; Himwich, Dravid & Berk, 1967; Roberts, 1967; Lowden & LaRamée, 1969). For these reasons we decided to use labelled amino acids as a more sensitive index of amino acid transport into the brain acid-soluble pool. Similar interference with the transport of some amino acids *in vivo* in hyperphenylalaninaemia has been observed by Guroff & Udenfriend (1962), Linneweh, Ehrlich, Graul & Hundeshagen (1963) and Roberts (1967), and *in vitro* by Neame (1961). To ascertain the effect of phenylalanine on an amino acid presumably not transported by the same carrier as phenylalanine (Neame, 1967, 1968) we examined the uptake of [^{14}C]glycine into the brain acid-soluble pool under

identical experimental conditions (Table 3). Unlike our findings with [^{35}S]methionine and [^{14}C]leucine, cerebral uptake of [^{14}C]glycine was unimpaired. It was concluded that phenylalanine interferes with the transport into the brain of only those amino acids for which a common carrier may exist (Blasberg & Lajtha, 1966).

Incorporation of amino acids into brain proteins in hyperphenylalaninaemia. In our experiments incorporation of ^{35}S -labelled and ^{14}C -labelled amino acids into brain proteins of the phenylalanine-treated animals was inhibited to a similar extent as was their transport into the acid-soluble pool. This suggests that elevated serum phenylalanine concentration decreases the rate of incorporation of [^{35}S]methionine and [^{14}C]leucine into protein by diminishing their entry into the brain acid-soluble pool. Incorporation into brain protein was not significantly impaired when [^{14}C]glycine was used as a precursor. Contrary to the proposals of Peterson & McKean (1969), there is no indication from our acute experiments that an increased brain phenylalanine concentration inhibits protein synthesis. In chronic conditions of hyperphenylalaninaemia or in phenylketonuria the amino acid imbalance may have an even more profound effect, for Roberts & Zomzely (1966) have shown that protein synthesis in the central nervous system is highly sensitive to changes in the internal environment, particularly to alterations in free amino acid concentrations.

The possibility cannot be ruled out that metabolites of phenylalanine exert the toxic effect instead of phenylalanine itself (Shah, Peterson & McKean, 1969), but, until we are able to determine in hyperphenylalaninaemia (a) brain phenylpyruvate and phenyl-lactate concentrations, (b) rate of conversion of phenylalanine into these metabolites in brain *in situ* and (c) serum phenylpyruvate and phenyl-lactate concentrations and the renal thresholds of these compounds, it will be reasonable to accept that phenylalanine is the toxic agent. Further, there was no inhibition of brain protein or lipid biosynthesis in acute experiments when we gave 18-day-old rats 0.2mg of phenylpyruvate and phenyl-lactate/g body wt. On the other hand, increasing the dose of phenylpyruvate to 2.0mg/g body wt. caused convulsions and ultimately the animals died within 4h (H. C. Agrawal, A. H. Bone & A. N. Davison, unpublished work).

Myelination and hyperphenylalaninaemia. In chronic hyperphenylalaninaemia, the concentrations of glutamate, aspartate, γ -aminobutyrate (Lowden & LaRamée, 1969) and some monoamines (Yuwiler & Geller, 1969) have been found to be lowered in the brain. Since these substances are considered to act as neurotransmitters or modulators within the central nervous system it is probable that changes in their concentration interfere with

neuronal function, and this may account for some of the mental deficiency frequently seen in phenylketonuria.

Since myelination is the last structural event in the ontogeny of the central nervous system it is reasonable to suggest that hyperphenylalaninaemia during the vulnerable period of myelination (Davison & Dobbing, 1966) exerts its deleterious effect primarily on the formation of the myelin sheath. During this period in the rat phenylalanine is a more effective inhibitor of myelin protein synthesis (Table 1 and Fig. 2). Work of Lowden, Clarke, Stockwell & LaRamée (1969) has established that phenylalanine interferes with the process of myelination. Thus in chronic hyperphenylalaninaemia deposition of myelin was decreased by about 60% in the rat brain. Similar decreases in the proteolipid protein content of the phenylketonuric brain have been reported by Prensky *et al.* (1968). Since we do not know the exact functional role of myelin in the central nervous system it is premature to suggest that interference with the deposition of myelin can lead to the decrease in intellectual performance. However, if complete myelination of the central nervous system in any species is a prerequisite for adequate intellectual performance, it is possible that inhibition of myelin protein synthesis during the vulnerable period of brain development may be one of the causes of mental retardation in phenylketonuria.

Thanks are given to Dr M. K. Gaitonde for amino acid analyses and Dr J. Daly and Dr J. W. T. Seakins for phenylalanine assays. We are grateful to Miss Alison Beard for technical assistance.

REFERENCES

- Agrawal, H. C., Bone, A. H. & Davison, A. N. (1969a). *Biochem. J.* **112**, 27P.
- Agrawal, H. C., Bone, A. H. & Davison, A. N. (1969b). *Proc. 2nd int. Meet. International Society for Neurochemistry, Milan*, p. 65.
- Agrawal, H. C., Davis, J. M. & Himwich, W. A. (1966). *J. Neurochem.* **13**, 607.
- Alvord, E. C., Stevenson, L. D., Vogel, F. S. & Engle, R. L. (1950). *J. Neuropath. exp. Neurol.* **9**, 298.
- Autilio, L. A., Norton, W. T. & Terry, R. D. (1964). *J. Neurochem.* **11**, 17.
- Barbato, L., Barbato, I. W. M. & Hamanaka, A. (1968). *Brain Res.* **7**, 399.
- Berl, S. & Frigyesi, T. L. (1967). *Proc. 1st int. Meet. International Society for Neurochemistry, Strasbourg*, p. 16.
- Blasberg, R. & Lajtha, A. (1966). *Brain Res.* **1**, 86.
- Carver, M. J. (1965). *J. Neurochem.* **12**, 45.
- Crome, L., Tymms, V. & Woolf, L. I. (1962). *J. Neurol. Neurosurg. Psychiat.* **25**, 143.
- Davison, A. N. & Dobbing, J. (1966). *Br. med. Bull.* **22**, 40.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Gaitonde, M. K. & Richter, D. (1956). *Proc. R. Soc. B*, **145**, 83.
- Gueroff, G. & Udenfriend, S. (1962). *J. biol. Chem.* **237**, 803.
- Hackney, I. M., Hanley, W. B., Davidson, W. & Linsao, L. (1968). *J. Pediat.* **72**, 646.
- Hall, T. C. & Cocking, E. C. (1965). *Biochem. J.* **96**, 626.
- Hess, H. H. & Lewin, E. (1965). *J. Neurochem.* **12**, 205.
- Himwich, W. A., Dravid, A. R. & Berk, T. J. C. (1967). *Regional Development of the Brain*. p. 221. Oxford: Blackwell Scientific Publications.
- Jervis, G. A. (1953). *Proc. Soc. exp. Biol. Med.* **82**, 514.
- Kaufman, S. (1958). *Science, N.Y.*, **128**, 1506.
- Linneweh, F., Ehrlich, M., Graul, E. H. & Hundeshagen, H. (1963). *Klin. Wschr.* **41**, 253.
- Lowden, J. A., Clarke, J. T. R., Stockwell, M. J. & LaRamée, M. A. (1969). *Proc. 1st int. Meet. International Society for Neurochemistry, Milan*, p. 274.
- Lowden, J. A. & LaRamée, M. A. (1969). *Can. J. Biochem.* **47**, 291.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McCaman, M. W. & Robins, E. (1962). *J. Lab. clin. Med.* **50**, 885.
- McKean, C. M., Boggs, D. E. & Peterson, N. A. (1968). *J. Neurochem.* **5**, 235.
- Malamud, N. (1966). *J. Neuropath. exp. Neurol.* **25**, 254.
- Menkes, J. H. (1966). *Pediatrics, Springfield*, **37**, 967.
- Menkes, J. H. (1967). *Pediatrics, Springfield*, **39**, 297.
- Menkes, J. H. (1968). *Neurology, Minneap.*, **18**, 1003.
- Mitoma, C., Auld, R. M. & Udenfriend, S. (1957). *Proc. Soc. exp. Biol. Med.* **94**, 634.
- Neame, K. D. (1961). *Nature, Lond.*, **192**, 174.
- Neame, K. D. (1967). In *Progress in Brain Research*, vol. 29, p. 185. Ed. by Ford, D. H. & Lajtha, A. Amsterdam: Elsevier Publishing Co.
- Neame, K. D. (1968). In *Applied Neurochemistry*, p. 119. Ed. by Davison, A. N. & Dobbing, J. Oxford: Blackwell Scientific Publications.
- Peterson, N. A. & McKean, C. M. (1969). *J. Neurochem.* **16**, 1211.
- Poser, C. M. & van Bogaert, L. (1959). *Brain*, **82**, 1.
- Prensky, A. L., Carr, S. & Moser, H. W. (1968). *Neurology, Minneap.*, **19**, 1003.
- Roberts, S. (1967). In *Progress in Brain Research*, vol. 29, p. 235. Ed. by Ford, D. H. & Lajtha, A. Amsterdam: Elsevier Publishing Co.
- Roberts, S. & Zomzely, C. E. (1966). In *Protides of the Biological Fluids*, vol. 13, p. 91. Ed. by Peeters, H. Amsterdam: Elsevier Publishing Co.
- Shah, S. N. & McKean, C. M. (1968). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **27**, 488.
- Shah, S. N., Peterson, N. A. & McKean, C. M. (1968). *Biochim. biophys. Acta*, **164**, 604.
- Shah, S. N., Peterson, N. A. & McKean, C. M. (1969). *Biochim. biophys. Acta*, **187**, 236.
- Swaiman, K. F., Hosfield, W. B. & Lemieux, B. (1968). *J. Neurochem.* **15**, 687.
- Wallace, H. W., Moldave, K. & Meister, A. (1957). *Proc. Soc. exp. Biol. Med.* **94**, 632.
- Woolf, L. I. (1963). *Adv. clin. Chem.* **6**, 134.
- Yuwiler, A. & Geller, E. (1969). *J. Neurochem.* **16**, 999.