The Effects of Hyperphenylalaninaemia on the Concentrations of Aminoacyl-Transfer Ribonucleic Acid *in vivo*

A MECHANISM FOR THE INHIBITION OF NEURAL PROTEIN SYNTHESIS BY PHENYLALANINE

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An acute administration of phenylalanine to neonatal animals has been reported to result in large decreases in the intracellular concentrations of several essential amino acids in neural tissue, as well as an inhibition of neural protein synthesis. The present report evaluates the effects of the loss of amino acids on the concentrations of aminoacyl-tRNA *in vivo*, with the view that an alteration in the concentrations of specific aminoacyl-tRNA molecules could be the rate-limiting step in brain protein metabolism during hyperphenylalaninaemia. tRNA was isolated from saline- and phenylalanine-injected mice 30-45 min after injection, by using a procedure designed to maintain the concentrations of aminoacyl-tRNA present *in vivo*. Periodate oxidation of the non-acylated tRNA and aminoacylation with radioactively labelled amino acids was used to determine the proportion of tRNA that was present *in vivo* as aminoacyl-tRNA. Although decreases in the intracellular concentrations of alanine, lysine and leucine were observed after phenylalanine administration, the concentrations of alanyl-tRNA, lysyl-tRNA and leucyl-tRNA actually increased by 15%. Although tryptophan has been suggested to be rate-limiting during hyperphenylalaninaemia, the proportion of tryptophan tRNA that was acylated was maximal in both normal and hyperphenylalaninaemic animals. This unexpected increase in aminoacyl-tRNA concentration is discussed as perhaps a secondary effect resulting from the phenylalanine-induced inhibition of protein synthesis. In contrast, the proportion of methionine tRNA that was acylated *in vivo* after phenylalanine administration was demonstrated to be decreased by approx. 17%. When the isoaccepting species of methionine tRNA were separated by reverse-phase column chromatography, three species were separated, one of which was demonstrated to be the initiator species, tRNA^{Met}, by the selective aminoacylation and formylation with *Escherichia coli* enzymes. After the administration of phenylalanine, the acylation of each of the three methionine tRNA species was decreased, with the initiator species being lowered by 10%. This effect on aminoacylation of tRNA^{Met} may be the primary step by which phenylalanine affects neural protein synthesis, and this is consistent with previous reports that re-initiation may be inhibited during hyperphenylalaninaemia.

The developing brain of humans and animals has been shown to be markedly affected by a chronic excess of phenylalanine in the blood circulatory system (Waisman *et al.*, 1960; Schlesinger *et al.*, 1969). Long-term excesses of phenylalanine have been associated with deficiencies in the total myelin content, both in the human phenylketonuric patient (Menkes, 1968; Shah *et al.*, 1972b), and in experimentally induced hyperphenylalaninaemia in animals (Prensky *et al.*, 1971; Shah *et al.*, 1972a). In addition to chronic conditions associated with phenylketonuria, acute administration of phenylalanine has been shown to affect brain protein metabolism. It has been demonstrated that the administration of phenylalanine causes 'disaggregation' of brain poly-

ribosomes (Aoki & Siegel, 1970; Siegel *et al.*, 1971; MacInnes & Schlesinger, 1971; Taub & Johnson, 1975), which results in an increase in 80S monoribosomes. This increase in 80S monoribosomes was shown to be the result of a decrease in the rate of initiation of brain protein synthesis, since the released ribosomes were vacant couples and not bound to mRNA or peptidyl-tRNA (Taub & Johnson, 1975). Although the balance between initiation and termination of polypeptide synthesis appeared to be shifted by the introduction of phenylalanine, the exact mechanism responsible for this phenomenon at the translational level was unknown.

In addition to the effect on brain polyribosomes, phenylalanine has been shown to cause a decrease

in the intracellular concentration of several amino acids. As demonstrated by McKean *et al.* (1968) and Hughes & Johnson (1976a), the exposure of neural tissue to high concentrations of phenylalanine lowered the concentrations of most other amino acids, and particularly, the large neutral amino acids, i.e. leucine, isoleucine, valine, tryptophan and methionine. The alterations in the concentrations of these amino acids, some of which are normally present at very low concentrations in neural tissue, has often been suggested as the primary reason for the inhibition of protein synthesis by phenylalanine (Appel, 1970; McKean *et al.*, 1968; Aoki & Siegel, 1970; Siegel *et al.*, 1971).

There have been suggestions that phenylalanine may interfere with the enzymic activation, and subsequent acylation, of other amino acids to tRNA (Roscoe *et al.*, 1968; Appel, 1970). An inhibition of the aminoacylation reaction might alter the concentrations of this essential precursor (aminoacyl-tRNA) for protein synthesis. The present investigation was directed towards a direct measurement of the effects of phenylalanine on the concentrations of several species of aminoacyl-tRNA *in vivo*, with the view that this might provide an explanation of the mechanism responsible for the inhibition of initiation events in neonatal brain tissue.

Materials and Methods

Materials

SCH:ARS(ICR)_r non-inbred Swiss albino mice were purchased from ARS/Sprague-Dawley (Madison, WI, U.S.A.). Non-radioactive amino acids were purchased from Nutritional Biochemical Co. (Cleveland, OH, U.S.A.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). ATP, CTP, NaIO₄, (±)-L-tetrahydrofolate and deoxyribonuclease (EP-1 from bovine pancreas) were also obtained from Sigma Chemical Co. Radioactive amino acids and [¹⁴C]-formate were purchased from New England Nuclear (Boston, MA, U.S.A.). Reverse-phase column-chromatography material (RPC-5) was obtained from Miles Laboratories (Elkhart, IN, U.S.A.).

Administration of phenylalanine

L-Phenylalanine, dissolved in 0.15M-NaCl to a final concentration of 30mg/ml, was prewarmed to 37°C before injection and was administered by intraperitoneal injection at a dose of 2mg of phenylalanine per g body weight. Control animals matched for age and weight (all animals in this study were 1-8 days old) were injected with a comparable volume of 0.15M-NaCl (60 µl/g body weight).

Preparation of aminoacyl-tRNA

tRNA was isolated from the injected animals between 30 and 45min after injection. This time period has been shown to be the period in which to observe the optimal effects of phenylalanine on brain protein synthesis (Taub & Johnson, 1975). Aminoacyl-tRNA was isolated by the procedure described by Allen *et al.* (1969), as modified by Johnson & Chou (1973). To minimize deacylation of aminoacyl-tRNA during the isolation, whole brains of Swiss albino mice were quickly removed and immediately placed into a Waring blender containing 1 vol. of sodium acetate buffer (150mM-NaCl/50mM-sodium acetate, pH 5.1) at 4°C, 1 vol. of phenol (saturated with the sodium acetate buffer) and 1mg of bentonite/ml. After the addition of each brain, the tissue was immediately homogenized and the final homogenate was centrifuged (2000g for 15min) to separate the phenol and aqueous phases. The aqueous phase was deproteinized further by two additional treatments with phenol (30ml) and finally with chloroform plus 1% (v/v) 3-methylbutan-1-ol (30ml). The nucleic acids from the final aqueous layer were precipitated with 2 vol. of ethanol at -20°C. Ribosomal RNA was removed from this nucleic acid fraction by precipitation with 1M-NaCl (5ml). The tRNA fractions were resuspended in sodium acetate buffer (2ml) and divided into two equal portions, one of which was treated with a 100-fold molar excess of NaIO₄ to oxidize the non-acylated tRNA. The other portion received distilled water and was to be used as a measure of total acceptor activity. To allow complete oxidation of the non-acylated tRNA, the samples were incubated for 15min, in the dark, at room temperature (25°C). The oxidized and control preparations were precipitated with ethanol and dialysed against glass-distilled water overnight. These were precipitated once again with 2 vol. of ethanol and deacylated by incubation for 3h at 37°C in Tris/HCl buffer, pH 9.0. The preparations were reprecipitated with ethanol, resuspended in a high-salt buffer [0.5M-NaCl/0.05M-MgCl₂/0.01M-Tris/HCl (pH 7.4)] (1ml), and 100 µg of deoxyribonuclease was added to remove any contaminating DNA in the solution. After a 2h incubation at room temperature, the aqueous layer was deproteinized with 1 vol. of phenol and 1 vol. of chloroform. After repeated precipitations to wash the tRNA, the solution was dissolved in a small volume (approx. 1ml) of water, and the A₂₆₀ was determined. The tRNA samples were stored at -20°C until their use for the measurement of amino acid-acceptor activity.

Aminoacyl-tRNA synthetases

Mouse brain aminoacyl-tRNA synthetases were prepared from the postmicrosomal fractions of

mouse brain tissue (Johnson & Chou, 1973). This fraction was concentrated by adding $(\text{NH}_4)_2\text{SO}_4$ to 75% saturation, and the precipitate sedimented by centrifugation (2000g for 15 min). The protein was dissolved in synthetase buffer [10mM-Tris/HCl (pH 7.4)/10mM-MgCl₂/6mM-2-mercaptoethanol] and filtered through a Sephadex G-75 column to remove the bulk of the endogenous tRNA (Johnson, 1969). The fractions containing protein were pooled and then dialysed overnight against the synthetase buffer. The amount of total protein present was determined by the colorimetric method of Lowry *et al.* (1951), with bovine serum albumin as a standard, and the synthetase preparations were stored at -85°C.

A crude preparation of *Escherichia coli* enzymes containing the aminoacyl-tRNA synthetases and the tRNA^{Met} transformylase were prepared by the procedure of RajBhandary & Ghosh (1969) with some modifications. An overnight culture of *E. coli* K12 (9g) was collected by centrifugation, washed with a solution containing 10mM-Tris/HCl (pH 8.0), 10mM-MgCl₂ and 10% (v/v) glycerol, and the cells were disrupted by using a French pressure cell (1040 MPa). This solution was then centrifuged at 100000g_{av} for 120 min. The supernatant was applied to a column (2.5cm×22cm) of DEAE-cellulose previously equilibrated with a buffer containing 20mM-potassium phosphate (pH 7.5), 20mM-2-mercaptoethanol, 10mM-MgCl₂ and 10% (v/v) glycerol, and the column was washed with the same buffer after the addition of all of the enzyme preparation. The mixture of synthetases was eluted with a solution containing 250mM-potassium phosphate (pH 6.5), 20mM-2-mercaptoethanol, 10mM-MgCl₂ and 10% glycerol. The fractions containing the peak of protein were pooled (120 ml), concentrated by using an Amicon filtration apparatus with a PM-10 filter and then dialysed overnight against a solution containing 2mM-potassium phosphate (pH 7.0), 40mM-2-mercaptoethanol and 10% glycerol. The total protein present was determined as above and the enzyme preparation was mixed with an equal volume of glycerol and stored at -85°C.

Aminoacylation of tRNA

The aminoacylation of tRNA was carried out in a reaction mixture (0.5 ml) containing 20mM-Tris/HCl, pH 7.2, 14.3mM-2-mercaptoethanol, 6μmol each of 19 non-radioactive amino acids, 0.35–1.3 A₂₆₀ units of tRNA, 0.18–0.32 mg of synthetase protein, 1–2μCi of ¹⁴C-labelled amino acids and the optimal concentrations of ATP, CTP and MgCl₂ as outlined in Table 1. The reaction mixtures were incubated for 40 min at 37°C and terminated by the addition of 2 vol. of ethanol and 10 mg of glycogen carrier. The precipitates were collected by centrifugation (1000g for 10 min), washed, reprecipitated twice and finally

collected on glass-fibre filters. The amount of radioactivity on each filter was determined in a Beckman LS-100 liquid-scintillation system (Hughes & Johnson, 1976a) with a counting efficiency of more than 95% for ¹⁴C.

The aminoacylation of mouse brain methionine tRNA with *E. coli* enzymes was carried out in a reaction mixture (0.5 ml) containing 20mM-Tris/HCl (pH 7.2), 5mM-MgCl₂, 1.25mM-ATP, 0.1mM-CTP, 19 non-radioactive amino acids, each at 6μM, 5 mg of *E. coli* enzymes, 0.5–1.0 A₂₆₀ unit of tRNA and 1μCi of [¹⁴C]methionine. The reaction mixture was incubated for 30 min at 37°C and then deproteinized by phenol and chloroform extraction and washed by ethanol precipitation as described above, ready for chromatography on the RPC-5 column.

Reverse-phase (RPC-5) column chromatography

Chromatography was performed as described by Kelmers & Heatherley (1971). tRNA from each preparation was acylated as described above, deproteinized by phenol and chloroform extraction and washed by repeated precipitation with ethanol. Approx. 5000–10000 c.p.m. of [¹⁴C]methionyl-tRNA in sodium acetate buffer was layered on the column (1.0cm×28cm), and the tRNA was eluted at 25°C by a 200 ml linear salt gradient (0.4–0.7M-NaCl) in a buffer consisting of 10mM-sodium acetate, 10mM-MgCl₂ and 2mM-2-mercaptoethanol (pH 4.5). Fractions (1.0 ml) were collected, and the amount of radioactivity in each fraction was determined as described above. The refractive index of various fractions was measured to determine the linearity of the gradient and the salt concentration at which each peak of [¹⁴C]methionyl-tRNA was eluted from each column. The recovery of radioactivity from the RPC-5 columns was 92–105%.

Preparation of 10-[¹⁴C]formyltetrahydrofolate

10-[¹⁴C]Formyltetrahydrofolate was prepared enzymically from [¹⁴C]formate and (±)-L-tetrahydrofolate with *Clostridium acidii-urici* formyltetrahydrofolate synthetase, which was kindly provided by Dr. Jesse C. Rabinowitz (University of California, Berkeley, CA, U.S.A.). The reaction mixture contained 0.10mCi of sodium [¹⁴C]formate in 4 ml of water, 3 ml of 10mM-(±)-L-tetrahydrofolate, 1.2 ml of 50mM-ATP, 1.2 ml of 0.1M-MgCl₂, 0.4 ml of 1M-KCl, 0.2 ml of 1M-triethanolamine buffer (pH 8.0) and 150 units of formyltetrahydrofolate synthetase. This was incubated for 20 min at 37°C and then diluted with 40 ml of 0.10M-2-mercaptoethanol and washed on to a DEAE-cellulose column (2.5 cm×10 cm) previously equilibrated with 0.05M-ammonium acetate (pH 8.0)/0.10M-2-mercaptoethanol. The 10-[¹⁴C]formyltetrahydrofolate was eluted from the

column with a linear gradient from 0.05 to 0.25M-ammonium acetate (pH8.0) in a buffer containing 0.10M-2-mercaptoethanol. The desired compound was eluted in the final fractions of the gradient, which were pooled, freeze-dried, resuspended in 0.10M-2-mercaptoethanol and freeze-dried again. The sample was dissolved in 0.10M-HCl and stored in a desiccator jar at -20°C as the stable 5,10-methenyl derivative. Small portions were diluted with 0.1M-Tris/HCl containing 0.2M-2-mercaptoethanol just before its use, and the pH was adjusted to 7.5 to convert the material into the 10-formyl derivative.

*Aminoacylation and formylation of mouse brain tRNA^{Met}**

The formylation of mouse brain tRNA^{Met} was performed essentially by the procedure described by Samuel *et al.* (1970). The reaction mixtures (0.5ml) included Tris/HCl (pH7.2) (20mm), MgCl₂ (5mm), ATP (2.5mm), ammonium acetate (10mm), L-methionine (0.2mm), *E. coli* enzymes, 5mg of protein 0.5–1.0 *A*₂₆₀ unit of mouse brain tRNA and 10-[¹⁴C]formyltetrahydrofolate, 0.5μCi. The mixture was incubated for 30min at 37°C, and then deproteinized by phenol and chloroform extraction and was washed by ethanol precipitation ready for reverse-phase chromatography on RPC-5 columns.

Results

Aminoacylation reaction conditions

The periodate-oxidation technique provides a reliable method for the determination of the portion of tRNA molecules which are present as aminoacyl-tRNA *in vivo*. The periodate-oxidation step of the tRNA isolation will oxidize the terminal ribose sugar residue of a non-acylated tRNA molecule, such that the tRNA molecule will no longer accept amino acids. After subsequent deacylation of the tRNA, the total amount of tRNA in the preparation can be determined through the use of radioactively labelled amino acids. The tRNA sample that was not treated with periodate provides a means of determining the total tRNA acceptor activity for each specific amino acid. From the proportion of total tRNA that is resistant to periodate oxidation can be estimated the concentration of precursor available as aminoacyl-tRNA (Allen *et al.*, 1969; Johnson & Chou, 1973).

*Abbreviations: tRNA^{Met}, non-acylated methionine tRNA species that can be formylated with *E. coli* enzyme, i.e. the initiator species; tRNA^{Met}, non-acylated methionine tRNA that cannot be formylated, i.e. the internal methionine tRNA; Met-tRNA^{Met} or Met-tRNA^{Met}, acylated isoaccepting species; fMet-tRNA^{Met}, the acylated and formylated methionine tRNA initiator species.

Since a study of the precursor tRNA concentrations is dependent on the acylation of all of the tRNA with radioactive amino acids, the optimal conditions for the aminoacylation reactions were first determined. This was particularly important, since previous work (Johnson & Chou, 1973) has shown that the optimal acylation of brain tRNA with various amino acids is dependent on the concentrations of ATP, CTP, Mg²⁺ and synthetase protein used in the reaction. Therefore appropriate concentrations of deacylated tRNA were incubated *in vitro* in the reaction mixtures, and the effect of various concentrations of ATP, CTP, Mg²⁺ and synthetase enzyme on the aminoacylation for each amino acid was tested. A summary (Table 1) of some of these data reveals some rather striking differences in the optimal conditions for the aminoacylation reaction for these amino acids. In particular, the Mg²⁺ concentration for maximum acceptance of lysine, leucine and alanine was 7-fold greater than that measured for tryptophan and methionine. In addition, the activities of individual mouse brain synthetases were determined for each amino acid used in this study, so that the concentration of synthetase enzyme was used at a saturating concentration. In a typical reaction, the amount of aminoacyl-tRNA formed was dependent only on the amount of tRNA added (Fig. 1).

Effects of phenylalanine on tRNA concentrations and amount of acylation

Preliminary results indicated that the total acceptor activity as well as the proportion of tRNA isolated as aminoacyl-tRNA differed considerably for each amino acid (Table 2). The aminoacylation reactions provided a comparison between the highly acylated methionyl-tRNA (91%) and the somewhat lower extent of acylation measured for alanine and lysine (each approx. 80%) for this single tRNA preparation.

Table 1. *Optimal conditions for acceptance of amino acids by mouse brain tRNA and aminoacyl-tRNA synthetase*

The standard reaction mixtures, as described in the Materials and Methods section, contained 0.4 *A*₂₆₀ unit of tRNA and 0.20mg of enzyme protein. MgCl₂, ATP and CTP were each titrated to determine the concentration of each which gave optimal amounts of aminoacylation for each individual amino acid.

Amino acid	MgCl ₂ (mm)	ATP (mm)	CTP (mm)
Lysine	10.0	0.75	0.10
Tryptophan	1.50	0.75	0.05
Leucine	10.0	1.00	0.10
Alanine	10.0	1.13	0.15
Methionine	1.00	0.60	0.10

Fig. 1. Aminoacylation of mouse brain tRNA

The aminoacylation reactions, as described in the Materials and Methods section, were used to determine the amount of (a) alanyl-tRNA, (b) lysyl-tRNA and (c) methionyl-tRNA present, by using the appropriate ¹⁴C-labelled amino acid. The amounts of total tRNA (●) and the periodate-resistant tRNA (○) were both determined for each tRNA preparation.

Table 2. Effects of phenylalanine on the amounts and the extent of acylation of various aminoacyl-tRNA species

The reaction mixtures, as listed in the Materials and Methods section, were used to determine the amount of aminoacyl-tRNA present for the periodate-resistant tRNA and the total tRNA present for one preparation from saline-injected animals and one preparation from phenylalanine-injected animals. The amount of tRNA present is expressed as pmol of aminoacyl-tRNA formed per A₂₆₀ unit of tRNA ± S.E.M., and the number of independent measurements for each individual preparation is indicated in parentheses.

Amino acid	Phenylalanine	Amount of aminoacyl-tRNA		Acylation (%)
		Total tRNA	Periodate-resistant tRNA	
Alanine	—	46.3 ± 0.90 (8)	35.9 ± 0.68 (8)	77.6
Alanine	+	49.5 ± 0.54 (11)	45.3 ± 0.72 (12)	91.5
Lysine	—	20.6 ± 0.74 (4)	16.6 ± 0.93 (4)	80.5
Lysine	+	18.3 ± 0.66 (6)	16.5 ± 0.34 (6)	90.2
Leucine	—	24.4 ± 0.72 (8)	21.1 ± 0.61 (10)	86.5
Leucine	+	30.2 ± 0.40 (6)	29.8 ± 0.99 (5)	98.6
Tryptophan	—	13.0 ± 0.53 (5)	13.8 ± 0.17 (8)	106.2
Tryptophan	+	21.5 ± 1.3 (8)	21.7 ± 0.75 (10)	101.0
Methionine	—	17.0 ± 0.26 (8)	15.5 ± 0.51 (6)	91.0
Methionine	+	18.8 ± 0.24 (6)	13.4 ± 0.16 (10)	71.3

tRNA preparations from the brains of animals injected with phenylalanine were compared with the tRNA isolated from control animals with respect to the amounts and extent of acylation of five different aminoacyl-tRNA species (Table 2). Although some differences in the tRNA-acceptance activity were detected in the different tRNA preparations, no significant decreases were detected in the amounts of total tRNA available for a specific amino acid after the administration of phenylalanine. However, when the proportions of tRNA available as aminoacyl-tRNA were compared, some very interesting differences were detected. These preliminary results were extended to include several preparations

of tRNA from young animals in order to measure the effects of phenylalanine on the concentrations of aminoacyl-tRNA *in vivo*.

In general, as indicated by the summary of Table 3, a state of hyperphenylalaninaemia produces an increase in the acylation of most amino acid tRNA species. This is particularly surprising, since high concentrations of phenylalanine actually decrease the intracellular concentrations of most of these amino acids in brain cells (McKean *et al.*, 1968; Hughes & Johnson, 1976a). Alanine and lysine, though not in the same transport class as phenylalanine, are decreased in their intracellular concentrations by about 15–20% after brain cells are

Table 3. *Summary of the effects of phenylalanine on the acylation of various amino acids*

The acylation of the tRNA preparations in control and in hyperphenylalaninaemic mice were determined as described in the Materials and Methods section. The values are presented as the mean % of the total tRNA isolated as aminoacyl-tRNA $\pm$ 1 s.d., with the numbers of independent tRNA preparations in parentheses where appropriate. When only two independent tRNA samples were tested for either the control or hyperphenylalaninaemic animals, then the mean is reported with actual values for individual preparations given in parentheses. Each of the preparations was assayed several times for tRNA-acceptor activity as demonstrated by the measurements listed in Table 2. Significance was determined by the Student's *t* test: **P* < 0.001; †*P* < 0.01.

Amino acid	Aminoacylation (%)		
	Control mice	Hyperphenylalaninaemic mice	Change
Alanine	75.0 $\pm$ 2.1 (4)	91.3 $\pm$ 1.5 (4)	+16.3*
Lysine	82.2 (80.5, 83.8)	96.7 (100, 100, 90.2)	+14.5
Leucine	83.7 (86.6, 80.8)	97.8 (97.0, 96.6)	+14.1
Methionine	94.2 $\pm$ 2.4 (3)	77.4 $\pm$ 4.9 (4)	-16.8†
Tryptophan	98.9 (100, 97.8)	94.8 (100, 89.5)	-4.1

exposed to high concentrations of phenylalanine. Yet the proportion of tRNA isolated as aminoacyl-tRNA was always higher in the animals injected with phenylalanine, suggesting that these amino acids may be more available for protein synthesis than they are in control animals.

The other three amino acids (Table 3) are considered to be in the same transport class as phenylalanine and are decreased considerably by the introduction of phenylalanine. Despite the loss of intracellular leucine under these conditions of hyperphenylalaninaemia, the concentration of leucyl-tRNA *in vivo* actually increases. A second large neutral amino acid, tryptophan, the intracellular concentration of which also decreases during hyperphenylalaninaemia, shows little or no alteration of the proportion of the tRNA that was acylated *in vivo*. Although a small decrease in acylation was detected, this may only be a reflection of the high proportion of tryptophan tRNA that is normally acylated in control animals (Tables 2 and 3). Thus, for the first four aminoacyl-tRNA species studied, either no significant change (tryptophan) or an increase in the aminoacyl-tRNA was observed (as with leucine, lysine and alanine), and the increase for the latter three amino acids was very similar.

Methionine tRNA was the only tRNA species studied that showed any significant decrease in its acylation *in vivo*. An average decrease of 17% of the amount of tRNA present in the aminoacylated form was demonstrated for all tRNA preparations from hyperphenylalaninaemic animals. This decrease was the only indication that the concentration of a direct precursor for protein synthesis was lowered in the animals injected with phenylalanine.

The decrease in the amounts of methionyl-tRNA may be of particular importance in the present study, since Met-tRNA^{Met} has been demonstrated to be an integral part of the initiation complex in both

prokaryotic and eukaryotic cells (Schreier & Staehelin, 1973; Gupta *et al.*, 1975; Smith & Henshaw, 1975). Since the study by Taub & Johnson (1975) suggested that the initiation of protein synthesis may be rate-limiting during hyperphenylalaninaemia, it appeared that the acylation of the initiator, tRNA^{Met}, could represent an important regulatory site of protein synthesis which might be altered by high concentrations of phenylalanine.

Separation of the isoaccepting species of methionine tRNA

Reverse-phase column chromatography was used to resolve the isoaccepting methionine tRNA species present in mouse brain tissue and to determine the extent of acylation of each isoaccepting species. The tRNA preparations from normal and hyperphenylalaninaemic mice were acylated essentially as described above, except that 3 ml of reaction mixtures was utilized to provide sufficient amounts of [¹⁴C]methionyl-tRNA for chromatography. The acylated tRNA was extracted with phenol and collected by repeated ethanol precipitation as described in the Materials and Methods section. The methionyl-tRNA species were separated by reverse-phase column chromatography by using RPC-5 (Kelmers & Heatherley, 1971). A typical separation of mouse brain methionyl-tRNA on RPC-5 by using a linear NaCl gradient to elute the tRNA from the column is shown in Fig. 2. Three discrete peaks of brain methionyl-tRNA were resolved by this procedure. This profile is similar to that observed for other eukaryotic systems by using this same chromatographic material (Caskey *et al.*, 1967; Yang *et al.*, 1969; Crystal *et al.*, 1972; Wang *et al.*, 1974).

The three isoaccepting methionine tRNA species were further characterized to determine which of these is the initiator species, tRNA^{Met}. Several

Fig. 2. Isoaccepting species of methionine tRNA separated by RPC-5 chromatography

[^{14}C]Met-tRNA from mouse brain was prepared as in the reaction described in the Materials and Methods section and then separated on a RPC-5 column by using a linear NaCl gradient from 0.4 to 0.7M. The small amount of radioactivity eluted in the void volume (tubes 10–20) of the column was free methionine. (a) Refractive index of each fraction; (b) radioactivity in each fraction.

Fig. 3. Mouse brain methionine tRNA acylated with either *E. coli* or mouse brain synthetase enzymes

Mouse brain tRNA was acylated with mouse brain synthetase and [^3H]methionine (●) or with *E. coli* synthetase and [^{14}C]methionine (○) as described in the Materials and Methods section. The two labelled tRNA preparations were then chromatographed on the same RPC-5 column by using a NaCl gradient from 0.4 to 0.7M. Only the fractions containing radioactively labelled methionyl-tRNA are shown.

investigators have reported that *E. coli* methionyl-tRNA synthetase enzymes can only acylate the tRNA^{Met} species and cannot acylate the internal methionine tRNA species that are present in eukaryotic cells (Bhaduri *et al.*, 1970; Crystal *et al.*, 1972; Stanley, 1972; Samuel *et al.*, 1973). When mouse brain tRNA was acylated with [^{14}C]methionine and *E. coli* enzymes, and then co-chromatographed with mouse brain tRNA acylated with [^3H]methionine and mouse brain synthetases, only the methionyl-tRNA present in the first peak eluted from the column (fractions 108–124) was acylated with the *E. coli* enzymes (Fig. 3). It appears that the first tRNA species eluted from the column is the initiator, Met-tRNA^{Met}, and the other species are the internal methionyl-tRNA molecules.

In addition, the initiator species Met-tRNA^{Met}, can be formylated by an *E. coli* transformylase, whereas the internal methionyl-tRNA species (Met-tRNA^{Met}) cannot be formylated. An *E. coli* enzyme preparation and 10-[^{14}C]formyltetrahydrofolate was utilized to formylate mouse brain tRNA, and this labelled tRNA was then co-chromatographed with

Fig. 4. Formylated methionyl-tRNA co-chromatographed with mouse brain methionyl-tRNA

Mouse brain tRNA was acylated with mouse brain enzymes and [^3H]methionine (●) or mouse brain methionyl-tRNA was formylated with *E. coli* enzymes and 10-[^{14}C]formyltetrahydrofolate (○) as described in the Materials and Methods section. The radioactively labelled preparations were then chromatographed on the same RPC-5 column by using a NaCl gradient from 0.4 to 0.7M.

Table 4. *Effects of phenylalanine on the acylation of the isoaccepting species of methionine tRNA*

The amount of aminoacyl-tRNA present in each of the three isoaccepting species of methionine tRNA was determined by RPC-5 column chromatography. The values for the amounts of tRNA for one representative tRNA preparation from control mice and one tRNA preparation from hyperphenylalaninaemic mice are presented and these amounts were determined by summing the amount of radioactivity eluted in each peak from the column per A_{260} unit of tRNA added to the aminoacylation reaction. Similar results for the acylation of each isoaccepting species were obtained from three different tRNA preparations for both groups and the summary of these are presented as the percentage acylation $\pm$ S.E.M.

Condition	Peak number	Total tRNA (c.p.m./ A_{260})	Periodate-resistant tRNA (c.p.m./ A_{260})	Acylation (%)	Summary of acylation*
Control	1	846	814	96.2	96.5 $\pm$ 2.4
	2	250	239	95.8	95.2 $\pm$ 1.0
	3	241	228	94.5	93.8 $\pm$ 0.9
	Total	1337	1281	95.8	
Hyperphenylalaninaemic	1	599	520	86.9	87.0 $\pm$ 1.5
	2	225	175	77.9	76.9 $\pm$ 5.4
	3	228	175	76.8	77.1 $\pm$ 5.2
	Total	1052	870	82.7	

* Each peak was found to be significantly different between the two tRNA groups by Student's *t* test and all were at $P < 0.05$.

mouse brain tRNA acylated with mouse synthetase enzymes and [3 H]methionine (Fig. 4). A single peak of mouse brain [14 C]fMet-tRNA^{Met} was eluted from the column at a higher salt concentration than the first isoaccepting methionyl-tRNA species. Since only tRNA that has been acylated with methionine can be formylated and *E. coli* enzymes can only acylate the methionine tRNA in the first peak from the RPC-5 column, it appears that the addition of a formyl group to Met-tRNA^{Met} has altered the chromatographic properties of this species. This alteration of the elution profile of Met-tRNA^{Met} after enzymic formylation to fMet-tRNA^{Met} has previously been reported by Samuel *et al.* (1973). It thus appears that the first species of mouse brain methionine tRNA eluted from the RPC-5 columns contains the initiator, tRNA^{Met}, since this is the only species that can be acylated and formylated by *E. coli* enzymes.

Effects of phenylalanine on the acylation of the isoaccepting species of methionine tRNA

The extent of acylation of each species of methionine tRNA was determined by separating the [14 C]Met-tRNA of the periodate-treated sample and the non-oxidized sample (total tRNA) of each tRNA preparation on individual RPC-5 columns. The amount of radioactivity eluted in each peak was summed, and from these results the amount of acylation of each isoaccepting species *in vivo* could be determined (Table 4). As expected from the acylation of the total Met-tRNA (Table 3), each of the three isoaccepting species of methionine tRNA in normal animals was highly acylated, usually 95–100%. The acylation of all

three isoaccepting species from tRNA preparations isolated after the administration of phenylalanine was decreased. The acylation of the initiator species, tRNA^{Met} (peak 1), was decreased by 9.5%, whereas the other internal methionine tRNA species were both decreased by approx. 17% (18.3% for peak 2, 16.7% for peak 3) from the values measured in control preparations of tRNA isolated from mice injected with 0.15M-NaCl.

Discussion

The acute administration of phenylalanine has been demonstrated to induce the loss of several amino acids from neural tissue (Aoki & Siegel, 1970; Siegel *et al.*, 1971; McKean *et al.*, 1968; Hughes & Johnson, 1976a). In particular, the amino acids that are co-transported with phenylalanine in brain tissue (i.e. leucine, isoleucine, methionine, tryptophan, tyrosine, valine) are most affected by high concentrations of phenylalanine. The large decreases in the intracellular concentrations of these amino acids may be explained by competition between phenylalanine and these other amino acids for transport into brain cells (Guroff & Udenfriend, 1962; Lindroos & Oja, 1971), as well as by phenylalanine influencing the efflux of these amino acids from brain cells (Hughes & Johnson, 1976a).

The altered intracellular concentrations of amino acids caused by hyperphenylalaninaemia has received much attention as a probable explanation for the inhibition of neural protein synthesis. Furthermore, as suggested by Appel (1970) and Roscoe *et al.* (1968), the aminoacylation of specific tRNA mole-

cules could be decreased because either the concentration of an amino acid is now limiting for aminoacylation or the high concentration of phenylalanine might inhibit the aminoacylation reaction. A decrease in the amount of aminoacyl-tRNA could affect the rate of donation of a specific amino acid to the growing polypeptide at the ribosomal level, which might result in a decrease in brain polyribosomes and therefore in neural protein synthesis.

Although the alteration of the intracellular amino acid concentrations by hyperphenylalaninaemia and the concomitant loss of protein synthesis have been well established, the effects on the aminoacylation of tRNA have been difficult to analyse. Cell-free experiments have suggested that phenylalanine can indeed decrease the aminoacylation of some tRNA molecules (Roscoe *et al.*, 1968). Until the development of the assays to determine the concentration of aminoacyl-tRNA in neural tissue *in vivo* (Johnson & Chou, 1973), it has been difficult to evaluate the effects of phenylalanine on the aminoacylation of tRNA *in vivo*.

Despite the decreases in amino acids in neural tissue induced by hyperphenylalaninaemia, the proportion of the tRNA isolated as aminoacyl-tRNA actually increased for the tRNA molecules specific for some amino acids. For instance, although administration of phenylalanine decreases the intracellular concentration of alanine and lysine by 10–20%, the proportion of tRNA present as aminoacyl-tRNA increases by 16.3% for alanine tRNA and 14.5% for lysine tRNA (Table 3). Such an increase in the amount of acylation is the exact opposite of that expected if the hypothesis presented above is correct.

Since several investigators have indicated that the amino acid pools for the large neutral amino acids (those co-transported with phenylalanine) are even more substantially decreased during hyperphenylalaninaemia, the acylation of some of these amino acids was also determined. Although phenylalanine induces a loss of 40–50% of the intracellular leucine from the brain of young animals, the concentration of leucyl-tRNA actually increased by 14.1% *in vivo* (Table 3). Even though the effect of phenylalanine on the intracellular concentration of leucine is different from that seen with alanine and lysine, the increases in the proportion of aminoacyl-tRNA for these three amino acids is very similar.

The amino acid tryptophan has been demonstrated by several investigators (Sidransky *et al.*, 1967; Pronczuk *et al.*, 1968) to play a special role in polyribosome formation and in maintenance of the normal rate of protein synthesis, since its deletion from an animal's diet results in disaggregation of liver polyribosomes. In addition, after an intraperitoneal injection of phenylalanine, the kinetics of the loss of tryptophan from neural tissue has been

demonstrated to correlate well with the breakdown of polyribosomes and the inhibition of protein synthesis (Siegel *et al.*, 1971). The importance of this amino acid in maintaining the normal rate of protein synthesis may be emphasized by its presence at very low concentration in neural tissue (McKean *et al.*, 1968). Therefore tryptophan could represent a rate-limiting amino acid, whose concentration might influence the rate of protein synthesis. The present study indicates that, even though the intracellular concentration of tryptophan may be decreased by phenylalanine administration, the proportion of the tryptophan tRNA that is acylated is relatively unchanged (Tables 2 and 3).

In the light of the lower intracellular concentrations of several amino acids during hyperphenylalaninaemia, it is surprising that the concentration of aminoacyl-tRNA actually increases. This suggests that the concentration of the precursor aminoacyl-tRNA molecules are not directly correlated with amino acid concentrations. It also suggests that the action of phenylalanine on the protein-synthetic mechanism is not at the level of the aminoacylation of these particular tRNA molecules.

Since high concentrations of phenylalanine are known to inhibit protein synthesis, the increase in the concentration of aminoacyl-tRNA may actually be a reflection of this inhibition. For example, if an inhibitor of protein synthesis, such as phenylalanine, decreases the rate of protein synthesis or the number of ribosomes initiating on to and translating an mRNA molecule, then the rate of donation of an amino acid to the growing polypeptide on a ribosome would also be decreased. This would probably result in an increase in the proportion of tRNA that is present intracellularly as aminoacyl-tRNA. Indeed, the increase in the amounts of leucyl-tRNA, alanyl-tRNA and lysyl-tRNA may only be a secondary effect, resulting from the inhibition of protein synthesis caused by high concentrations of phenylalanine.

The concentration of methionyl-tRNA is, however, decreased during hyperphenylalaninaemia (Tables 2 and 3) with an average loss of 17%. In fact, when the methionyl-tRNA molecules are separated into the three isoaccepting species by reverse-phase chromatography, it was determined that high concentrations of phenylalanine decreased the acylation of all three methionine tRNA species. This suggested that high concentrations of phenylalanine may indeed affect the normal aminoacylation of all methionine tRNA species. The effect on the aminoacylation reaction *in vivo* might be due to either the methionine concentration falling to a rate-limiting value and or to high concentrations of phenylalanine directly inhibiting the synthetase specific for the acylation of methionine tRNA.

The effect of high concentrations of phenylalanine on the acylation of methionine tRNA is of particular importance because of the special role of methionyl-tRNA in cellular protein synthesis. In particular, one isoaccepting species, $\text{tRNA}_f^{\text{Met}}$, is involved in the initiation complex for protein synthesis (Schreier & Staehelin, 1973; Gupta *et al.*, 1975; Smith & Henshaw, 1975) and its intracellular concentration could possibly regulate the rate of protein synthesis.

The initiator species, $\text{tRNA}_f^{\text{Met}}$, was identified in mouse brain tissue by the selective acylation and formylation of this single methionine tRNA species (Figs. 3 and 4) with *E. coli* enzymes. The selective acylation and formylation of the initiator, as well as the shift in elution pattern of the initiator after formylation, has been reported previously by several investigators for many different eukaryotic cell types (Crystal *et al.*, 1972; Samuel *et al.*, 1973; Stanley, 1972). It thus appears that in mouse brain tissue the initiator, $\text{tRNA}_f^{\text{Met}}$, is eluted first from the RPC-5 column, which is also consistent with the result of other investigators with eukaryotic cell types, using this chromatographic material (Crystal *et al.*, 1972; Wang *et al.*, 1974; Yang *et al.*, 1969).

In animals made hyperphenylalaninaemic, the concentration of acylated initiator, Met- $\text{tRNA}_f^{\text{Met}}$, was demonstrated to be decreased by 9.5% (Table 4). The decrease in the acylation of $\text{tRNA}_f^{\text{Met}}$ is noteworthy in the light of the results of Taub & Johnson (1975), which suggested that the phenylalanine-induced 'disaggregation' of polyribosomes may be due to a lowered rate of re-initiation of ribosomes. Thus the decrease in the aminoacylation of the tRNA species specific for the initiation of protein synthesis might be the site at which phenylalanine decreases the normal rate of protein synthesis.

The acylation of the other two isoaccepting species of methionine tRNA is also decreased by high concentrations of phenylalanine, apparently to an even greater extent, with an 18.3% (peak 2) and a 16.3% (peak 3) loss in aminoacyl-RNA concentration. Although the reason for the differential sensitivity of the isoaccepting species to high concentrations of phenylalanine is presently unknown, it does seem clear that the aminoacylation of all species of methionine tRNA is affected during hyperphenylalaninaemia.

From the results presented in the studies by Taub & Johnson (1975), Hughes & Johnson (1976a) and in the present study, a model could be proposed to explain the inhibition of protein synthesis detected after the administration of phenylalanine, which would include the following elements. (1) An alteration of the intracellular concentrations of most amino acids, and, in particular, a decrease in methionine and an increase in phenylalanine; (2) a decrease in the aminoacylation of methionine tRNA, and, especially, $\text{tRNA}_f^{\text{Met}}$, resulting from

either lowered methionine concentrations or increased phenylalanine concentrations that might be inhibitory to the aminoacylation reaction; (3) a decrease in initiation and in the number of ribosomes normally present in polyribosomes owing to a decrease in the amount of acylated $\text{tRNA}_f^{\text{Met}}$; (4) a decrease in the normal rate of protein synthesis; (5) this decrease in the rate of synthesis would decrease the normal rate of aminoacyl-tRNA turnover, such that there would be an increase in the proportion of other tRNA molecules present as aminoacyl-tRNA (such as alanyl-tRNA, leucyl-tRNA etc.). This model suggests that methionine and $\text{tRNA}_f^{\text{Met}}$ may become rate-limiting for protein synthesis during hyperphenylalaninaemia. The model is supported by the demonstration that the 9.5% decrease in the acylation of the initiator, $\text{tRNA}_f^{\text{Met}}$, is compatible with the results of Taub & Johnson (1975), in which it was reported that about 10% of the polyribosomes are disaggregated to monoribosomes as a result of a decreased rate of initiation.

Although Roberts & Morelos (1976) have suggested that brain polyribosome changes during hyperphenylalaninaemia may be a reflection of increased ribonuclease activity, the present study, along with the observations Taub & Johnson (1975), clearly shows that ribonuclease is not responsible. In addition, the hypothesis that brain polyribosome disaggregation is a reflection of an amino acid imbalance, as described above, is supported by the re-formation of brain polyribosomes after the administration of a mixture of amino acids that includes methionine (Hughes & Johnson, 1976b).

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