The effect of elevated plasma phenylalanine levels on protein synthesis rates in adult rat brain

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Increasing the plasma phenylalanine concentration to levels as high as 0.560–0.870 mM (over ten times normal levels) had no detectable effect on the rate of brain protein synthesis in adult rats. The average rates for 7-week-old rats were: valine, 0.58 ± 0.05 %/h, phenylalanine, 0.59 ± 0.06 %/h, and tyrosine, 0.60 ± 0.09 %/h, or 0.59 ± 0.06 %/h overall. Synthesis rates calculated on the basis of the specific activity of the tRNA-bound amino acid were slightly lower (4% lower for phenylalanine) than those based on the brain free amino acid pool. Similarly, the specific activities of valine and phenylalanine in microdialysis fluid from striatum were practically the same as those in the brain free amino acid pool. Thus the specific activities of the valine and phenylalanine brain free pools are good measures of the precursor

specific activity for protein synthesis. In any event, synthesis rates, whether based on the specific activities of the amino acids in the brain free pool or those bound to tRNA, were unaffected by elevated levels of plasma phenylalanine. Brain protein synthesis rates measured after the administration of quite large doses of phenylalanine (> 1.5 $\mu\text{mol/g}$) or valine (15 $\mu\text{mol/g}$) were in agreement (0.62 ± 0.01 and 0.65 ± 0.01 %/h respectively) with the rates determined with infusions of trace amounts of amino acids. Thus the technique of stabilizing precursor-specific activity, and pushing values in the brain close to those of the plasma, by the administration of large quantities of precursor, appears to be valid.

INTRODUCTION

The hypothesis that elevated levels of phenylalanine in the plasma can inhibit brain protein synthesis rates has been the subject of numerous reports over several decades. Many of these studies were directed toward investigating phenylketonuria (PKU), but it was recognized that acutely elevated levels of phenylalanine are not the best model for PKU and more recent work employs long-term elevation of phenylalanine levels. Although these studies are not all in agreement (see e.g. Agrawal et al., 1970; Aoki and Siegel, 1970; Hughes and Johnson, 1977; Roberts, 1977; Binek and Johnson, 1982), a number of authors concluded that acute loading with phenylalanine inhibited brain protein synthesis rates, but usually only at very high plasma levels (greater than 1–2 mM) and only in young animals. However, a recent report (Wall and Pardridge, 1990) suggests that even modest increases (to 4-fold of normal, or 0.216 mM) in plasma levels produced large (25%) inhibitions in adult rat brain protein synthesis rates. Such an effect would have implications not only for the consumption of free phenylalanine, and possibly other amino acids, which are readily available to consumers in the U.S.A., but also for procedures using large doses of amino acids for the measurement of protein synthesis rates. As these findings are at variance with work done by us, and others, we have conducted additional studies aimed at clarifying this question. Here we present data on the brain protein synthesis rate measured by constant infusion of radioactively labelled amino acid via an implanted catheter in the presence of either normal or elevated levels of phenylalanine. Rate measurements were also carried out after the injection of large doses of either labelled valine or labelled phenylalanine.

MATERIALS AND METHODS

Materials

Escherichia coli tRNA, amino acids and chloral hydrate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H.p.l.c.-grade solvents, inorganic salts and acids, and solvents for organic extraction, came from J. T. Baker Inc. (Phillipsburg, NJ, U.S.A.) or Fisher Scientific (Fairlawn, NJ, U.S.A.). Vanadyl ribonucleoside complex (VRC) was from GIBCO BRL (Gaithersburg, MD, U.S.A.) and phenol was from United States Biochemical (Cleveland, OH, U.S.A.). Labelled amino acids were obtained from Amersham (Arlington Heights, IL, U.S.A.). Nucleobond AX500 cartridges were from Macherey-Nagel (Duren, Germany). Poly(tetrafluoroethylene) tubing came from AIN Plastics (Mt. Vernon, NY, U.S.A.) and polyethylene tubing (Intramedic) from Clay Adams (Parsippany, NJ, U.S.A.). High-speed glass centrifuge tubes, Corex, were obtained from Corning Inc. (Corning, NY, U.S.A.). The stereotaxic instrument was made by David Kopf Instruments (Tujunga, CA, U.S.A.). The microdialysis equipment was purchased from Bioanalytical Systems (W. Lafayette, IN, U.S.A.). The h.p.l.c. system, and the desalting resin, were obtained from Bio-Rad (Richmond, CA, U.S.A.).

The animals used were males of the Sprague–Dawley strain, raised in the animal quarters on the premises, weighing 230–240 g, which corresponds to an age of about seven weeks.

Methods

The anaesthetic used was chloral hydrate at 0.5 ml/100 g of a 7.6% (w/v) solution (38 mg/100 g body wt., intraperitoneally).

A small incision was made in the middle of the back and then a 2-cm-long incision on the left side of the neck. The catheters were then pulled through under the skin and filled with a solution of heparin (1000 units/ml). With the animal supine, head toward the investigator, the left carotid artery, between the omohyoid and sternohyoid muscles, was teased out from the neck incision and exposed (about 5 mm). A suture (triple 0, cotton) was inserted under the artery rostrally and tied but not trimmed. The artery was then closed with a microclip as caudal as possible and another suture was inserted under the isolated artery and tied loosely. A small incision was then made in the artery with the point of a pair of fine scissors, and the end of the catheter was inserted into the artery and through the suture, which was then lightly tied. The artery clip was removed and blood observed to flow easily through the tubing. The tubing was then worked into the artery to a depth of 7–10 mm and the suture tied again, tightly, and trimmed. Another suture was used to secure the tubing in the artery. The anterior ligature, used to close the artery initially, was then also tied around the exposed rostral end of the catheter to secure it against movement. The left jugular vein was exposed and a catheter similarly inserted, except that no clip was used. The incisions were sutured and the catheters tied with the sutures to secure them to the skin on the back. The catheters were polyethylene (PE 20, Intramedic) for the artery and 28-gauge Teflon tubing for the jugular vein. After surgery, the rat was placed in a large bowl (part of the infusion system) with food and water *ad libitum* and allowed to recover for at least 2 days. A plastic band was placed around the animal's chest and attached to a wire running to a weighted lever arm on a pivot, affixed to the top of the bowl so that a slight tension was placed on the wire to keep it taut. The catheters were then connected to a dual-channel liquid swivel attached to the lever arm. In 5–10% of the rats the line for blood clogged before the experiment. Infusing heparin, 1000 units/ml, at a low rate (e.g. 0.1 $\mu\text{l}/\text{min}$) seemed to reduce this complication.

On the day of the experiment a solution of labelled amino acid was infused into the vein at a rate of 5–30 $\mu\text{l}/\text{min}$, depending on the desired level of plasma phenylalanine, for periods of either 1 or 2 h (1 h only at the highest dose). The solution contained labelled valine (usually L-[3,4(*n*)- ^3H]valine, 25–50 Ci/mmol) and labelled phenylalanine (usually L-[2,3,4,5,6- ^3H]phenylalanine, 100–130 Ci/mmol), with or without unlabelled phenylalanine (170 mM), in saline or water respectively. The amount of radioactivity used varied, but the amount of labelled amino acid administered was always inconsequential. In general both valine and phenylalanine were infused in amounts of 180–225 $\mu\text{Ci}/\text{h}$, though in some early experiments as little as 22–44 $\mu\text{Ci}/\text{h}$ of valine was infused. However, for experiments involving isolation of tRNA or microdialysis, 2 mCi of each amino acid were administered (about 0.010 $\mu\text{mol}/\text{h}$ of labelled phenylalanine and 0.040 $\mu\text{mol}/\text{h}$ of labelled valine). It was not possible to achieve the highest level of phenylalanine studied by infusion alone, so an intraperitoneal injection of 1.5 μmol of phenylalanine/g body weight (150 mM, in water) was given immediately before beginning the infusion of phenylalanine. The concentration and specific radioactivity of phenylalanine in both solutions was the same. Blood samples were withdrawn at the times indicated during the infusion. At the end of the infusion the animal was decapitated and the brain, after its temperature was measured, was removed and processed.

In some rats an arterial catheter was implanted as above, and then labelled valine was injected intraperitoneally at a dose of 15 $\mu\text{mol}/\text{g}$ body weight (500 mM, in water). Blood and tissue samples were taken as described.

Blood was centrifuged, and the plasma removed and mixed

with an equal volume of 6% (w/v) perchloric acid (PCA). After centrifugation the supernatant was stored frozen for subsequent amino acid analysis.

For the determination of the specific activity in the free and protein-bound amino acid pools of the brain, it was immediately homogenized in 3% (w/v) PCA and stored frozen. For processing, the homogenate was thawed, resuspended and centrifuged. The supernatant was removed and stored frozen for amino acid analysis. The pellet was resuspended in the same homogenizer and extracted, twice with 5% trichloroacetic acid, once with methanol, twice with chloroform/methanol (1:1, v/v) and once with ether. In each case the volume of solvent was 5 ml. After drying in air, several milligrams of the dry material were hydrolysed in a Teflon-capped tube (6 M HCl, 110 $^{\circ}\text{C}$ for 24 h). The hydrolysate was dried under vacuum and stored at -20°C for amino acid analysis.

Amino acid analyses were done by ion-exchange chromatography using a commercial column (Pickering Lab., Mountainview, CA, U.S.A.) with post-column fluorescence detection with *o*-phthalaldehyde (OPA) (Benson and Hare, 1975). Where greater sensitivity was needed, naphthylcarbamoyl derivatives of the amino acids were prepared (Neidle et al., 1989), separated by reversed-phase h.p.l.c. and detected by monitoring the fluorescence. In either case the column effluent was collected in 0.6–1.0 ml samples, mixed with 4.0 ml of Lquiscint scintillation fluid (National Diagnostics, Manville, NJ, U.S.A.) and analysed by liquid-scintillation spectrophotometry (Beckman Instruments, Palo Alto, CA, U.S.A.). Norleucine was used as an internal standard for ion-exchange analyses, and both norleucine and *p*-fluorophenylalanine for analyses with naphthyl derivatives. Tritiated leucine was also added as an internal standard for liquid-scintillation counting.

For determinations of the specific radioactivity of the tRNA-bound amino acid, tRNA was isolated by a variation of the procedure of Smith (1991). The brain was removed and homogenized in 10 ml of ice-cold 0.25 M sucrose containing 10 mM VRC, to inhibit ribonuclease, and 2 mg of *E. coli* tRNA, to act as carrier. (Some samples of carrier tRNA were unusable as they produced naphthyl derivatives which co-eluted with those of tyrosine and valine.) A 1 ml aliquot of the homogenate was mixed with an equal volume of 6% (w/v) PCA and reserved for specific radioactivity determinations of free and protein-bound amino acids. The remainder was centrifuged at 100 000 *g* for 1 h and 0.5 ml of trichloroacetic acid (100 g/100 ml) added to the supernatant to precipitate the aminoacyl-tRNA. The precipitate, obtained by centrifugation at 12 000 *g* for 20 min, was washed three times to remove free amino acids, and then resuspended in 9 ml of 0.3 M sodium acetate buffer, pH 5.5. To extract protein an equal volume of a solution of 1 mg/ml 8-hydroxyquinoline (acting as an antioxidant) in buffer-saturated phenol was added. After vigorous shaking at room temperature the mixture was centrifuged at 3000 *g* for 15 min and the aqueous layer removed and extracted with an equal volume of chloroform. It was then extracted twice more with ether to remove traces of phenol. This solution was applied to a silica-based anion-exchange cartridge, Nucleobond AX 500, equilibrated with 10 ml of buffer 'N' [100 mM Tris/phosphate buffer, pH 6.3, containing 15% (v/v) ethanol] at a flow rate of 1 ml/min. Free amino acids and proteins are not retained. The RNA adhering to the column was washed with 20 ml of the same buffer, N, but containing 500 mM KCl, and then eluted with high-salt buffer (N containing 1150 mM KCl) as a sharp peak visible on the u.v. monitor (see Figure 1). The eluent containing the RNA was collected in glass tubes (Correx) and the RNA precipitated with 0.8 vol. propan-2-ol and 1% (v/v) 1 M MgCl_2 . After incubation at -20°C for

Figure 1 Purification of aminoacyl-tRNA on an ion-exchange cartridge (Nucleobond AX500) and analysis of the amino acids released from the RNA fraction

(a) The cartridge was washed with a low-salt buffer, N, and then the sample, S, applied in 0.3 M sodium acetate (see Materials and methods section). Proteins and free amino acids, the contaminants of interest, washed through the column with the washing buffer, NW, while the RNA, including aminoacyl-tRNA, was retained. The RNA was then eluted with a buffer containing more salt, N3 (N plus 500 mM KCl), and the cartridge was washed with a high-salt buffer, N4, to remove tightly retained material, such as DNA. After equilibration with about 20 ml of N, a second sample was eluted from the same cartridge in this example. Flow rates were about 1 ml/min. Absorbance was measured at 290 nm, rather than 265 nm, because of the large amounts of RNA eluted. (b) The amino acids bound to the tRNA were released by incubation in borate buffer, pH 9.4, for 90 min at 37 °C and separated by precipitation of the RNA. Naphthyl derivatives were prepared and resolved on a C18 column. This sample contains 117, 31 and 33 pmol of valine, tyrosine and phenylalanine respectively.

45 min the precipitated RNA was pelleted by centrifugation (20 min, 12000 g). The pellet was resuspended in 0.5 ml of a solution composed of 1 vol. of 0.3 M sodium acetate, pH 5.5, mixed with 2.5 vol. of ethanol containing 0.12 M potassium acetate and transferred to a microfuge tube. The Corex tube was washed with 0.5 ml, and then 0.1 ml, of the same solution and the washings combined with the sample and centrifuged (15 min at

12000 g). The pellet, containing the aminoacyl-tRNA, was processed or stored frozen.

The pellet containing the aminoacyl-tRNA was dissolved in 100 μ l of 50 mM Na_2CO_3 and incubated at 37 °C for 90 min to release the amino acids. The carbonate solution was neutralized with 6% (w/v) PCA (about 10 μ l) and the tRNA precipitated by the addition of 100 μ l of 0.3 M sodium acetate, pH 5.5, and 0.8 vol. of propan-2-ol. After 20 min at -20 °C, the sample was centrifuged (12000 g for 15 min) and the supernatant, containing the free amino acids, removed and dried under vacuum. The amino acids were converted into naphthyl derivatives (Neidle et al., 1989) for amino acid analysis (see Figure 1), followed by liquid-scintillation counting. (Two commercial filters were tested for separating the tRNA from the amino acids, but, even with repeated washing, they introduced background peaks with the naphthyl isocyanate derivatization.)

Microdialysis probes, with a 3 mm dialysing surface (CMA10, Bioanalytical Systems), were used for sampling amino acids from the extracellular space. The animal was anaesthetized and placed in position on a stereotaxic instrument. The scalp was retracted, and a small hole drilled 0.7 mm rostral to the bregma and 2.5 mm left of the midline. A second hole was also drilled 5 mm to the left of and 2 mm rostral to, and a third 5 mm caudal to, the guide hole for positioning of anchoring screws. Spacers were attached to the guide to reduce the distance penetrated from the surface of the skull to 4 mm so that the end of the guide would penetrate about 0.5 mm into the striatum. This places the 3 mm dialysing surface of the probe, when inserted, entirely in the (left) striatum (see the stereotaxic atlas of Paxinos and Watson, 1982). (The actual position of the probe was confirmed in preliminary studies.) The guide was glued in place with dental cement and then catheters implanted as usual. On the day of the experiment, a probe was inserted through the guide and a modified Krebs-bicarbonate buffer (Butcher and Hamberger, 1987) pumped through the dialysis probe at a rate of 3 μ l/min for a 2 h equilibration period. (Phosphate was omitted because it produces a background peak with naphthyl isocyanate.) After the infusion of label into the vein had begun, microdialysate was collected over 15-min-long periods and reserved for amino acid analysis. When the brain was removed, the left and right striata were dissected and homogenized separately in 200 μ l of 3% (w/v) PCA. Otherwise the remainder of the brain, and the two striatal samples, were treated as described above. When probes were implanted for a prolonged period, no guide was used. The probe was positioned with the stereotaxic instrument and glued in place. A tethering system (Instech, Plymouth Meeting, PA, U.S.A.) was modified to extend the anchoring screw which was then glued to the skull, so that the lines from the probe could run inside the tethering spring to prevent them becoming dislodged. Fluid was infused through the probe at 0.1 μ l/min till the day of the experiment.

Data analysis

The specific radioactivities of the plasma amino acids were plotted against time for each animal. The curve-fitting program in Sigmaplot 5.0 (Jandel Scientific, San Raphael, CA, U.S.A.) was used to fit a curve to the data according to the following equation:

$$S = a \cdot [1 - \exp(-b \cdot t)]$$

where S is the specific radioactivity in the plasma and t is time of the infusion. The constant ' a ' corresponds to the plasma specific radioactivity at equilibrium, i.e. the maximum value. The area under the curve, determined with the transform function, divided

Figure 2 Typical plasma amino acid concentration curves after the administration of various amounts of phenylalanine

Labelled valine and labelled phenylalanine were administered at a constant rate with an infusion pump through a catheter in the jugular vein. For phenylalanine loading, the same solution, almost saturated with phenylalanine, was dispensed at a flow rate sufficient to give the load indicated, with the exception that for the highest levels it was necessary to give an intraperitoneal injection of phenylalanine at the beginning of the infusion. Blood was removed through a catheter in the carotid artery at various times during the infusion and the amino acid concentrations in the plasma were determined. (a) Rat no. 11, control infused with label only; (b) rat no. 21, infused with 0.23 μmol of Phe/g per h; (c) rat no. 27, infused with 0.66 μmol of Phe/g per h; and (d) rat no. 54, infused and injected with Phe.

by the time of infusion is the average specific radioactivity over the interval, S_{av} . The specific radioactivity in the brain was calculated by multiplying the average value for plasma by the ratio of either brain free: plasma, or brain tRNA-bound: plasma, specific radioactivities at the end of the experiment. This assumes that the shape of the specific activity curve for precursor in the brain is the same as that in the plasma, i.e. there is no lag in the brain versus plasma specific activity ratio. The synthesis rate, ' K_s ', was calculated as:

$$K_s = [S_{pro}/(S_{av} \cdot t)] \cdot 100$$

where K_s is the synthesis rate (in %/h), S_{pro} and S_{av} are the specific radioactivities of the protein bound and precursor amino acid respectively (in d.p.m./nmol) and t is time (in h).

RESULTS

In a preliminary experiment we considered whether there were gross differences in the d.p.m./mg of protein obtained per μCi infused for valine, leucine, tyrosine and lysine. Tyrosine (like phenylalanine) appeared to give considerably higher specific radioactivities in plasma, brain and brain protein, but tyrosine would be diluted in these experiments. None of the other amino acids was markedly more efficient than the rest. Valine was selected for the precursor to be used in low concentrations.

When unlabelled phenylalanine was included in the infusion, its concentration in the plasma increased with time, as shown in Figure 2, depending on the rate of infusion. The plasma phenylalanine level was elevated into three different ranges by: (1) infusing 0.23 $\mu\text{mol/g}$ per h (170 mM at 5 $\mu\text{l/min}$); (2) infusing 0.66 $\mu\text{mol/g}$ per h (170 mM at 15 $\mu\text{l/min}$); or (3) infusing 1.2 $\mu\text{mol/g}$ per h (150 mM at 30 $\mu\text{l/min}$) and also injecting 1.5 $\mu\text{mol/g}$ at the beginning of the infusion for a total of 2.7 $\mu\text{mol/g}$ over the 1 h period. It was necessary to use both an

injection and an infusion of phenylalanine to achieve plasma levels in the range of 0.5–0.8 mM. The dose used for the injection, 1.5 μmol of phenylalanine/g, was chosen so that the total amount of phenylalanine administered was well in excess of the amount used in a well-known loading procedure (Garlick et al., 1980). The plasma phenylalanine level begins to decline soon after the injection (Figure 2) so these experiments were limited to 1 h, when levels were highest. By these techniques, phenylalanine in the plasma was elevated to ranges of 0.117 (0.099–0.136), 0.225 (0.219–0.235) and 0.682 (0.559–0.869) mM respectively. Plasma phenylalanine levels in controls averaged 0.058 mM. (Food was available at all times and none of the data are fasting plasma levels.) Plasma tyrosine also increased from 0.053 mM to approx. 0.072, 0.134 and 0.427 mM respectively as a result of the administration of phenylalanine. Also shown are the plasma concentrations of several other amino acids which are transported by the same carrier as phenylalanine. The concentrations of a number of these amino acids were lower in the presence of elevated phenylalanine (Table 1), but the differences were not statistically significant.

The levels of free amino acids in the brain at the end of the infusion period were also altered by the increased plasma phenylalanine (Table 1). With the highest dose of phenylalanine, the level of that amino acid in the brain increased from 0.055 $\mu\text{mol/g}$ in controls to 0.279 $\mu\text{mol/g}$. The brain concentration of tyrosine rose also, from 0.050 to 0.284 $\mu\text{mol/g}$. On the other hand, the brain concentrations of isoleucine and leucine were reduced by this dose of phenylalanine, probably because of competition for carrier in the transport system.

The yields of amino acids bound to tRNA are shown in Table 1(c). They range from 117 pmol/g for leucine and valine to 35 pmol/g for tyrosine. As expected, the concentrations are not affected by changes in plasma or brain levels. To estimate the extent of recovery, aminoacyl-tRNA of high specific activity,

Table 1 Amino acid concentrations at the end of the infusion in plasma, brain and in brain aminoacyl-tRNA

Treatment consisted of the administration of phenylalanine through a catheter in the jugular vein plus, for the highest dose, an intraperitoneal injection of phenylalanine. Labelled amino acids were infused into the catheter in the jugular vein at a constant rate, while blood was withdrawn through a catheter in the carotid artery. Plasma, deproteinized with PCA, was analysed for amino acid content by ion-exchange chromatography with post-column fluorescence detection with OPA. Only the plasma concentrations at the end of the infusions are shown in Table 1. The brain was removed and homogenized at the end of the infusion in sucrose. A portion of the homogenate was mixed with PCA and the supernatant used for amino acid analysis as above. The rest of the homogenate was processed for preparation of aminoacyl-tRNA as described in the Materials and methods section. The amino acids were released from the tRNA by incubation in borate at pH 9.4 and subjected to amino acid analysis derivatives (Needle et al., 1989). All values are means $\pm$ S.D. The number of samples, *n*, is given in parentheses. With Student's *t* test, one tail: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with controls.

Amino acid	Amino acid concentrations			(b) In brain (nmol/g of brain)			(c) From aminoacyl-tRNA (pmol/g of brain)		
	(a) In plasma (nmol/ml of plasma)			Control	0.66 μ mol/g per h	2.7 μ mol/g	Control	0.66 μ mol/g per h	2.7 μ mol/g
	Control	0.23 μ mol/g per h	0.66 μ mol/g per h						
Valine	141 $\pm$ 31 (5)	164 $\pm$ 36 (7)	148 $\pm$ 17 (4)	72 $\pm$ 14 (5)	70 $\pm$ 20 (6)	57 $\pm$ 10 (4)	104 $\pm$ 13 (3)	137 $\pm$ 9 (2)	117 $\pm$ 21 (5)
Methionine	48 $\pm$ 15 (5)	46 $\pm$ 8 (7)	39 $\pm$ 3 (4)	21 $\pm$ 13 (4)	10 $\pm$ 8 (4)	34 $\pm$ 9 (4)	14 $\pm$ 6.4 (2)	6.0 $\pm$ 4.2 (2)	8.0 $\pm$ 6.5 (6)
Isoleucine	69 $\pm$ 17 (5)	82 $\pm$ 16 (7)	75 $\pm$ 9 (4)	37 $\pm$ 9 (5)	32 $\pm$ 11 (4)	25 $\pm$ 9 (4)*	85 $\pm$ 18 (3)	102 $\pm$ 4 (4)	95 $\pm$ 20 (3)
Leucine	132 $\pm$ 37 (5)	164 $\pm$ 28 (7)	123 $\pm$ 20 (4)	68 $\pm$ 15 (5)	55 $\pm$ 14 (6)	41 $\pm$ 8 (4)**	111 $\pm$ 31 (3)	123 $\pm$ 6 (4)	114 $\pm$ 198 (3)
Tyrosine	49 $\pm$ 13 (5)	83 $\pm$ 24 (7)**	172 $\pm$ 52 (4)***	45 $\pm$ 9 (5)	106 $\pm$ 30 (6)**	284 $\pm$ 96 (4)***	36 $\pm$ 5 (3)	36 $\pm$ 6 (3)	32 $\pm$ 5 (4)
Phenylalanine	63 $\pm$ 11 (5)	137 $\pm$ 21 (7)***	248 $\pm$ 16 (4)***	47 $\pm$ 10 (5)	173 $\pm$ 32 (6)***	279 $\pm$ 58 (4)***	38 $\pm$ 14 (3)	43 $\pm$ 6 (3)	37 $\pm$ 8 (3)

obtained from incubated brain slices (Dunlop et al., 1975b), was used as a tracer. The yields for valine, leucine, tyrosine and phenylalanine were 24 %, 22 %, 18 % and 18 % respectively. The amounts recovered (Table 1) are similar to those reported by Smith et al. (1991b) (valine, 91; isoleucine, 66; leucine, 106; tyrosine, 38; and phenylalanine, 21 pmol/g of brain). Keen et al. obtained a much higher yield of leucine, 0.6 nmol/g (Keen et al., 1989).

While the yields of amino acids from tRNA are only 35 to 117 pmol/g, if the values from the single recovery experiment are correct the amounts in tRNA would be 0.51, 0.53, 0.19 and 0.22 nmol/g for valine, leucine, tyrosine and phenylalanine respectively. Synthesis consumes the same amino acids at rates of 5.5, 7.1, 3.3 and 2.0 nmol/min per g. Thus the half-lives would be 3.9, 3.1, 2.4 and 4.6 s. Airhart and co-workers (1974) previously calculated the turnover time of valine in liver tRNA as 12 s and Keen et al. (1989) reported a half-life of 4.2 s in brain for leucine. The pools are thus very small and turn over very rapidly, which greatly increases the possibility of introducing errors into the measurement. Thus despite the development of new procedures, measurements of tRNA specific activity are still inherently difficult and expensive.

As amino acids were infused into the rat, the specific radioactivities in the plasma increased rapidly for 30–45 min and then stabilized. Examples of typical plasma specific radioactivity curves for valine and phenylalanine are shown in Figure 3.

When the specific radioactivities of the plasma amino acids are compared with those of the brain free amino acids, determined at the end of the experiment, the ratios were about 38 % for valine and 64 % for phenylalanine in controls (Table 2). As the dose of phenylalanine is increased, the ratio increases for phenylalanine until, at 2.7 μ mol/g, the brain specific activity is 90 % of the plasma value. The ratio for valine actually decreases in the presence of high levels of plasma phenylalanine to about 24 % with the highest load.

The specific radioactivities of the phenylalanine released from the aminoacyl-tRNA were slightly higher than those found in the homogenate, as shown in Table 3. It should be noted that when various amounts of phenylalanine were administered, the ratio of phenylalanine specific radioactivity in tRNA to that of the free amino acid in brain was practically unchanged, even at the highest level of phenylalanine.

The specific activities of the tRNA-bound amino acids could differ from those of the brain free amino acid pool for many reasons, including the preferential use of amino acids from one or more intracellular (e.g. lysosomal) or extracellular pools. We decided to determine the specific activity in the extracellular pool as this can be sampled by microdialysis. As shown in Figure 4, the specific activities of valine and phenylalanine in the microdialysis fluid from the striatum were found to be very similar to those in the free amino acid pools of the left and right striata or in whole brain. There is a marked increase in the phenylalanine concentration in the microdialysis fluid to about 1.59 pmol/ μ l at the end of the infusion, 4.5-fold the level seen in the control (0.35 $\pm$ 0.05 pmol/ μ l). The increase in the brain concentration was 4.0-fold. Plasma tyrosine increased from 0.48 nmol/ml to only 0.98 nmol/ml in 2 h, considerably less than in other rats infused with 0.66 μ mol/g per h. The tyrosine concentration increased by a similar amount: from 0.33 pmol/ μ l to 0.52 pmol/ μ l, in the microdialysate, and increased in the brain to roughly double that in the control.

Synthesis rates were calculated using the specific activities of the brain free amino acids. There were no differences between rates measured after 1 or 2 h of infusion and the data are summarized in Figure 5. Rates are shown for valine, phenyl-

Figure 3 Typical curves of plasma specific activity with time during the administration of various amounts of phenylalanine

Labelled valine and labelled phenylalanine, along with unlabelled phenylalanine, were administered with an infusion pump through a catheter in the jugular vein. For the highest level of phenylalanine an injection of phenylalanine was also given. Blood samples were withdrawn at various times during the infusion and the specific activities of valine, phenylalanine and, where feasible, tyrosine were determined in the plasma. Key to symbols: ■, plasma phenylalanine; □, brain phenylalanine; ●, plasma valine; ○, brain valine; ▼, plasma tyrosine; ▽, brain tyrosine. Panels represent the same infusion conditions as described in Figure 2.

Table 2 Specific activities of amino acids in brain compared with those in plasma

Specific activities were determined by collecting and counting the effluent from the amino acid analyses which were carried out using the ion-exchange column and detection with OPA (as described in the Methods section). The radioactivity present in each peak was measured with a liquid-scintillation spectrophotometer. All values given are means $\pm$ S.D. The number of samples (n) is given in parentheses.

Phenylalanine dose	Specific activity in brain compared with level in plasma (%)					
	Valine		Phenylalanine		Tyrosine	
	1 h	2 h	1 h	2 h	1 h	2 h
Control	38 $\pm$ 5 (6)	38 $\pm$ 5 (5)	61 $\pm$ 5 (5)	67 $\pm$ 6 (2)	73 (1)	62 (2)
0.23 μ mol/g per h	35 $\pm$ 9 (3)	42 $\pm$ 2 (4)	79 $\pm$ 3 (2)	89 $\pm$ 3 (2)	—	—
0.66 μ mol/g per h	32 (1)	31 $\pm$ 4 (4)	89 (1)	83 $\pm$ 7 (4)	73 (1)	74 (1)
2.7 μ mol/g	24 $\pm$ 7 (4)	—	90 $\pm$ 3 (4)	—	90 $\pm$ 5 (4)	—

alanine and, in some cases, for tyrosine. Also shown are the relative rates from the publication by Wall and Pardridge (1990) setting controls equal to 100% of the rate found here. With the perfusion system, the inhibitions found at plasma concentrations of 0.216, 0.432 and 0.864 mM phenylalanine were 25, 49 and 73% respectively, which would easily have been detected in these experiments. The average of all the rates measured with valine and phenylalanine using the infusion procedure was 0.59 ± 0.06 %/h. Similarly a linear regression of the synthesis rate versus plasma phenylalanine level gave an intercept of 0.59 %/h with a slope of -2.9×10^{-6} . The synthesis rate, within

95% confidence limits, at a plasma concentration of 0.800 mM is between 0.54 and 0.64 %/h. Brain protein synthesis rates were measured also using a dose (intraperitoneal injection) of 15 μ mol of valine/g body wt. as a loading or flooding dose, and, as shown in Figure 5, the rates measured were similar (0.65 ± 0.01 %/h) to those obtained with trace concentrations of valine or phenylalanine, or with high doses (> 1.5 μ mol/g) of phenylalanine (0.62 ± 0.01 %/h). Thus even at these very high levels, there is no detectable inhibition of brain protein synthesis rates with acute loads of either of these two amino acids.

The synthesis rates can also be calculated on the basis of the

Table 3 Specific activities of amino acids in tRNA compared with those in brain

Specific activities were determined by collecting and counting the effluent from the amino acid analyses carried out on a reversed-phase column after the preparation of naphthyl derivatives (as described in Table 1). The radioactivity present in each peak was measured with a liquid-scintillation spectrophotometer. Values are means $\pm$ S.D. The number of samples (*n*) is given in parentheses.

Phenylalanine dose	Specific activity (% of that in brain)		
	Val	Phe	Tyr
Control	97 $\pm$ 15 (3)	107 $\pm$ 10 (3)	73 (1)
0.66 μ mol/g per h	93 $\pm$ 4 (2)	101 $\pm$ 9 (3)	90 $\pm$ 15 (3)
2.7 μ mol/g	—	103 $\pm$ 7 (3)	102 $\pm$ 16 (3)
Average	96 $\pm$ 11 (5)	104 $\pm$ 8 (9)	93 $\pm$ 17 (7)

specific activities of the tRNA-bound amino acids, with the same assumptions used for the brain free amino acids. However, it is obvious from Table 3 that all the rates would be decreased slightly (by from 3 to 7% for phenylalanine) and that there is no difference in synthesis rates at different plasma phenylalanine levels. In fact, the relative rate at the highest level of phenylalanine

would be increased 2–4% relative to controls. There is then no indication of inhibited synthesis rates in brain at elevated plasma levels when tRNA-bound amino acid specific radioactivities are used to calculate the rates.

DISCUSSION

There has been a recent report (Wall and Pardridge, 1990) suggesting that even modest elevations of plasma levels of phenylalanine inhibit protein synthesis in adult rat brain. It was also suggested that the inhibition might be related in a linear fashion to concentration, rather than being dependent on a threshold level, so that any elevation would produce some inhibition. In the study presented here, we find no evidence of inhibition of brain protein synthesis rates in adult rats even at plasma phenylalanine levels of 0.560–0.870 mM. This is so whether the synthesis rate is based on the levels of the brain free amino acids or the tRNA-bound amino acids.

There are several factors which may be relevant to these different findings. First, the experimental systems employed are quite different. In the studies by Wall and co-workers (Hargreaves-Wall et al. 1990; Wall and Pardridge, 1990) the rat, still anaesthetized, received a 'retrograde infusion through the right external carotid artery' with an artificial blood containing

Figure 4 The specific radioactivities and concentrations of amino acids in the extracellular fluid and plasma as sampled by microdialysis during the infusion of labelled amino acids with and without a load of phenylalanine

Labelled valine and phenylalanine, with or without unlabelled phenylalanine, were given through a catheter in the left jugular vein with an infusion pump. Samples of microdialysis fluid were obtained during the infusion by pumping an artificial CSF through a microdialysis probe inserted through a guide into the (left) striatum. Samples of blood were also withdrawn through a catheter in the left carotid artery during the infusion. Amino acid concentrations and specific radioactivities were determined by amino acid analysis after derivatization with naphthyl isocyanate. Panels (a) and (c) show results obtained from rat no. 47 which was a control, and panels (b) and (d) show results from rat no. 48 which was infused with 0.66 μ mol/g per h. Key to symbols in (a) and (b): (■—■), Phe in plasma; (■···■), Phe in microdialysate; (●—●), Val in plasma; (●···●), Val in microdialysate; □, Phe in brain; ○, Val in brain.

Figure 5 Brain protein synthesis rates in the presence of elevated levels of plasma phenylalanine

Brain protein synthesis rates, measured by infusion, are shown for phenylalanine (○), valine (●) and tyrosine (▽). The synthesis rates are plotted against the average concentration of phenylalanine in the plasma over the course of the infusion. The solid line is a linear regression of synthesis rates with valine and phenylalanine versus plasma phenylalanine levels, and the broken lines show the 95% confidence levels. Note that the highest dose of phenylalanine is examined only over the first hour when the plasma concentration is highest. For comparison, synthesis rates measured with a flooding dose (intraperitoneal injection) of valine (□) are shown at the right margin. The apparent inhibition previously reported (Wall and Pardridge, 1990) is indicated by the dotted line. With infusions of 0.22 or 0.66 μmol of phenylalanine/g per h for 2 h, plasma levels ranged between 99 and 136 and between 219 and 235 nmol/ml respectively. With infusions of 0.66 μmol /g per h over 1 h plus an intraperitoneal injection of 1.5 μmol of phenylalanine/g, plasma levels were between 559 and 869 nmol/ml. In control infusions, plasma phenylalanine ranged from 50 to 72 nmol/ml.

labelled leucine. With a perfusion rate of 1 ml/min, the rate of brain protein synthesis was very slow (Hargreaves-Wall et al., 1990). But with a perfusion rate of 1.5 ml/min, the synthesis rate increased to 2.7 nmol of leucine/min per g of brain. Given that brain contains approx. 100 mg of protein/g (Pitts and Quick, 1967) and that protein contains about 750 nmol of leucine/mg of protein (Cornish-Bowden, 1983; Dunlop, 1983), this rate equals 0.22%/h, far below the rate of 0.6%/h (depending on age) obtained by a number of investigators with a variety of techniques (Dunlop et al., 1977; Goldspink, 1988; Smith et al., 1991b). The perfusion system also differs in that the specific activity of free leucine in the brain at equilibrium is only 38% of the value in the plasma, compared with 46% in infusions in awake, alert rats (Sun et al., 1992). These low values, particularly for protein synthesis rate, suggest that this perfusion system, even with a flow rate of 1.5 ml/min, is still not representative of brain tissue *in vivo*.

Also, it would appear that those rate measurements are based on the specific activity in the tissue only at 15 min, rather than over the entire interval of incorporation. Elevated levels of phenylalanine in the plasma could inhibit the transport of labelled leucine into the brain, so that the specific activity in the brain, especially in the early stages of the infusion, might be reduced.

The most crucial factor in the divergent findings appears to be the specific activity of the tRNA-bound amino acids. Wall and co-workers found that the level of the tRNA-bound precursor was equal to that of free leucine in controls (0.054 mM plasma phenylalanine), but that at 0.216 mM plasma phenylalanine, it was only 60% of the value for leucine in the tissue, while at 0.864 mM plasma phenylalanine, it was over 100% greater than that of the brain free leucine. In fact, if synthesis rates had been calculated on the basis of the levels in the free amino acid pool,

there would have been relatively little effect from the elevated plasma phenylalanine. We did not find any relative change at high phenylalanine levels. The specific activity of tRNA-bound phenylalanine was always within 7% of the value for the acid-soluble phenylalanine (Table 3). We can offer no explanation for this disparity. Smith and co-workers also reported no changes in specific activity of tRNA-bound valine (112% versus 104%) comparing controls with flooding levels of valine (Smith et al., 1991b).

When labelled valine was given at doses of 15 μmol /g body wt. i.e. 'flooding', there was similarly no detectable inhibition of brain protein synthesis (Figure 5). Smith et al., using an infusion system, also reported that synthesis rates in controls and rats flooded with valine were similar (Smith et al., 1991a), i.e. 5.2 and 5.5 nmol/g per min (about 0.54 and 0.57%/h using 100 mg of protein/g of brain and 580 nmol of valine/mg of protein). Thus, in adult rat brain, protein synthesis rates are not inhibited by gross elevations of either phenylalanine or valine. Elevations of this magnitude are routinely used to measure protein synthesis rates (Dunlop et al., 1975a; Garlick et al., 1980) in brain and other tissues.

Phenylalanine at the highest dose did alter the brain concentrations of some amino acids other than tyrosine and phenylalanine. The concentrations of isoleucine and leucine in the brain, compared with those in controls, are significantly lower (Table 1), which is consistent with previous reports (e.g. Antonas and Coulson, 1975; Smith et al., 1991b) with regard to the effects of high plasma levels of one amino acid on the brain concentrations of others. Valine, at the loading dose, reduced plasma concentrations of most of the other amino acids markedly, particularly those of tyrosine and phenylalanine (27 ± 4 and 27 ± 9 nmol/ml respectively at 60 min).

It has long been recognized that, with constant infusion, the specific activity in the brain reaches a plateau which is well below that in the plasma, and that the ratio of brain to plasma specific activity is a direct measure of recycling. Garlick and Marshall, in 1972, found that in mice the brain to plasma specific activity ratio of tyrosine approached 60%, and pointed out that this indicated that 60% of the tyrosine incorporated into brain protein was from plasma, while 40% must be from recycling. Our data (Table 2) indicate that recycled valine and phenylalanine constitute about 62 and 36% respectively of the free amino acid incorporated into brain proteins. The value for valine is similar to that previously published for adult rat brain (Smith et al., 1991b). It is also possible to estimate, from this ratio, the overall flow rate of essential amino acids from plasma into brain. If we calculate the rate of release of unlabelled amino acid from the degradation rate, which essentially equals the synthesis rate (using the average of all rates with infusions of valine and phenylalanine of 0.58%/h), and the known amino acid content of brain protein (Dunlop, 1978), we obtain rates of 5.5, 7.1, 3.3 and 2.0 nmol/min per g released from protein for valine, leucine, phenylalanine and tyrosine. As the specific activity ratios for brain versus plasma are 40, 46 (Sun et al., 1992), 51 and 34% respectively, then the effective flow rates from plasma to brain should be 3.4, 6.0, 3.2 and 3.9 nmol/min per g of brain.

Many attempts have been made to compare the specific activities of amino acids in tRNA to those in the tissue free amino acid pools, but the results have been mixed. Recently, brain tissue has been examined in this respect (Keen et al., 1989; Hargreaves-Wall et al., 1990; Smith et al., 1991b). With the possible exception of leucine, it appears that the levels of the tRNA-bound amino acids are almost equal to those of the brain free amino acid pool. Smith found that, with an infusion of valine, the specific activity of the brain free pool was very close to that of the tRNA-bound pool. The ratio of tRNA-bound to brain free levels at 60 min was 106%, and, for pooled data at 60 and 90 min, 114%. With valine loading, the ratios were 101% and 95% at 60 and 90 min respectively, where equilibrium had been reached. Similarly, with phenylalanine, we find the specific activity ratios (Table 3) to be 107% in controls and 101 to 103% with elevated plasma phenylalanine levels. It is interesting to note that whenever the precursor plasma concentration is elevated, as in loading procedures, the levels in the tRNA-bound and brain free pools are all within a few percent of each other. This is not surprising as, under these conditions, the specific activity in the brain is close to that in the plasma (phenylalanine = 90%, tyrosine = 90%, and, for valine loading, $90 \pm 2\%$). However, with an infusion of leucine in trace concentrations, the specific activity of the tRNA-bound leucine was found to be 118% of that of the brain free pool (Smith et al., 1988; Sun et al., 1992). With this exception, there seems to be agreement then that the specific activities of the tRNA-bound and of the brain free pool are equivalent measures of the precursor specific activity.

In regard to the ratio of the specific activities of valine in brain and blood after loading, Smith et al. have reported a lower value, 74% (Smith et al., 1991b). This may be due to differences in procedure as they load with unlabelled valine before beginning the infusion of label.

Measurements of specific activities in the extracellular fluid of two rats (Figure 4), as sampled by microdialysis, found valine to be only 6 and 11% higher, and phenylalanine to be 4 and 1% higher, than the specific activity in the brain free pool. The specific activities in the plasma pool are about 2–3 times the values in brain (Table 2). These samples were obtained from striatum, and specific activities could vary by region, but the

extent of the differences seen here suggests that the relatively small extracellular space, in intimate contact with the cells, is in rapid, essentially complete, equilibrium with the intracellular space. This being the case, the specific activity of the intracellular precursor pool (tRNA) presumably could not possibly be higher unless there were a mechanism to transport amino acids from the plasma directly to the intracellular space.

Alternatively, probes do damage the tissue, and it could be that the sample is drawn from a pool which is not actually the same as extracellular fluid. We implanted probes for two days to determine whether this period of healing might alter the relative specific activity. (It is known that after 3 or 4 days, the probe may be subject to gliosis and yields may drop.) We found that by 2 days the relative specific activity of valine in the microdialysate was considerably below that of the brain free amino acid pool. The microdialysis procedure was therefore carried out in the usual manner as described. We suggest that microdialysis fluid is probably a reasonable approximation of the extracellular fluid, particularly for specific activity measurements at equilibrium. As the limiting value, the specific activity in the extracellular fluid indicates that the levels of valine and phenylalanine in the tRNA are at most 8 and 2% higher respectively than that of the brain free pool. In principle protein synthesis rates could be measured by monitoring the specific activity in microdialysis fluid, but it would be a costly approach and, from these limited data, it appears that the specific activity in the microdialysate rises more slowly than that in the plasma.

As tRNA is acylated within the cells and the turnover rate is in seconds (Airhart et al., 1974), it seemed reasonable to assume that the source of amino acids for protein synthesis was the intracellular pool, and most synthesis rates in the literature have been calculated on that basis for many years. Although there was considerable debate on the possible effects of compartmentation on precursor specific activity, data were available to support the intracellular hypothesis. For example, the rate in young adult rat brain was determined with the valine-loading procedure (Dunlop et al., 1975a) to be 0.65%/h, and because the procedure pushes brain specific activity close to that in the plasma, this rate is established within a relatively narrow range. When measurements by a number of investigators with a variety of techniques were compared (Dunlop et al., 1977), rates calculated on the basis of the specific activity in the brain free pool were in good agreement with each other, and with the rate measured with high levels of valine, whereas rates calculated on the basis of the plasma specific activity were not only inconsistent but also were lower than the rate with high valine, which is presumably impossible. Recent work confirms and extends these conclusions by demonstrating that the specific activity in the tRNA is indeed close to that in the free amino acid pool, at least for valine and phenylalanine, and by demonstrating similar limits through measurements of the specific activities in the extracellular pool.

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REFERENCES

- Agrawal, H. C., Bone, A. H. and Davidson, A. N. (1970) *Biochem. J.* **117**, 325–331
- Airhart, J., Vidrich, A. and Khairallah, E. A. (1974) *Biochem. J.* **140**, 539–548
- Antonak, K. N. and Coulson, W. F. (1975) *J. Neurochem.* **25**, 309–314
- Aoki, F. and Siegel, F. L. (1970) *Science* **168**, 129–130
- Benson, J. R. and Hare, P. E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 619–622
- Binek-Singer, S. P. and Johnson, T. C. (1982) *Biochem. J.* **206**, 407–414
- Butcher, S. P. and Hamberger, A. (1987) *J. Neurochem.* **48**, 713–721
- Cornish-Bowden, A. (1983) *Biochem. J.* **213**, 271–274
- Dunlop, D. S. (1978) Measuring protein synthesis and degradation rates in CNS tissues. *Res. Methods Neurochem.*, pp. 91–141, Plenum Press, New York

- Dunlop, D. S. (1983) in *Handbook of Neurochemistry*, 2nd edn., pp. 25–63, Plenum Press, New York
- Dunlop, D. S., van Elden, W. and Lajtha, A. (1975a) *J. Neurochem.* **24**, 337–344
- Dunlop, D. S., van Elden, W. and Lajtha, A. (1975b) *Brain Res.* **99**, 303–318
- Dunlop, D., Lajtha, A. and Toth, J. (1977) in *Mechanisms, Regulation and Special Functions of Protein Synthesis in the Brain* (Roberts, S., Lajtha, A. and Gispen, W. H., eds.), pp. 79–96, Elsevier/North-Holland, New York
- Garlick, P. J. and Marshall, I. (1972) *J. Neurochem.* **19**, 577–583
- Garlick, P. J., McNurlan, M. A. and Preedy, V. R. (1980) *Biochem. J.* **192**, 719–723
- Goldspink, D. F. (1988) *J. Neurochem.* **50**, 1364–1368
- Hargreaves-Wall, K. M., Buciak, J. L. and Pardridge, W. M. (1990) *J. Cerebral Blood Flow Metab.* **10**, 162–169
- Hughes, J. V. and Johnson, T. C. (1977) *Science* **195**, 402–404
- Keen, R. E., Barrio, J. R., Huang, S. C., Hawkins, R. A. and Phelps, M. E. (1989) *J. Cerebral Blood Flow Metab.* **9**, 429–445
- Neidle, A., Banay-Schwartz, M., Sacks, S. and Dunlop, D. S. (1989) *Anal. Biochem.* **180**, 291–297
- Paxinos, G. and Watson, C. (1982) *The Rat Brain in Stereotaxic Coordinates*, Academic Press, New York
- Pitts, F. N. and Quick, C. (1967) *J. Neurochem.* **14**, 561–570
- Roberts, S. (1977) in *Mechanisms, Regulation and Special Functions of Protein Synthesis in the Brain* (Roberts, S., Lajtha, A. and Gispen, W. H., eds.), pp. 1–20, Biomedical Press, North Holland and Elsevier, New York
- Smith, C. B. (1991) *Neurochem. Res.* **16**, 1037–1046
- Smith, C. B., Deibler, G. E., Eng, N., Schmidt, K. and Sokoloff, L. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9341–9345
- Smith, C. B., Sun, Y., Deibler, G. and Sokoloff, L. (1991a) *Trans. Am. Soc. Neurochem.* **22**, 205
- Smith, C. B., Sun, Y., Deibler, G. E. and Sokoloff, L. (1991b) *J. Neurochem.* **57**, 1540–1547
- Sun, Y., Deibler, G. E., Sokoloff, L. and Smith, C. B. (1992) *J. Neurochem.* **59**, 863–873
- Wall, K. M. and Pardridge, W. M. (1990) *Biochem. Biophys. Res. Commun.* **168**, 1177–1183