Cerebral ribosomal protein phosphorylation in experimental hyperphenylalaninaemia

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Investigations were carried out on the effects of phenylalanine loading on ribosomal protein phosphorylation in cerebral cortices of infant rats. Administration of L-phenylalanine intraperitoneally, in doses of 1 or 2 mg/g body wt., resulted within 30 min in a significant decrease in incorporation of radioactivity from intracisternally administered [^{32}P]P_i into constitutive ribosomal proteins of the cerebral 40S subunit. This phenomenon was not accompanied by significant variations in ^{32}P uptake into the cerebral cytosol. Incorporation of radioactivity into ribosomal proteins of the cerebral 60S subunit exhibited only minor variations under these circumstances. Alterations in the phosphorylation state of cerebral 40S ribosomal proteins induced by phenylalanine loading involved principally the S6 protein, which exists in multiple states of phosphorylation. The proportions of the more highly phosphorylated congeners of this protein were markedly decreased, as detected by two-dimensional electrophoretograms and autoradiographs of the cerebral 40S ribosomal proteins. Phenylalanine loading also altered the relative extent of phosphorylation of the S6 protein in cerebral polyribosomes and monoribosomes. In control animals, the specific radioactivity of 40S proteins in cerebral polyribosomes was five to ten times that of 40S proteins in the monoribosome population. At 1 h after phenylalanine administration, the specific radioactivities of 40S proteins in the two ribosome populations tended to approach equality. These alterations in ribosomal protein phosphorylation were accompanied by a decrease in the proportion of polyribosomes in purified ribosome preparations isolated from cerebral cortices of phenylalanine-treated infant rats. In animals given the higher dose of phenylalanine (2 mg/g body wt.), subsequent administration of a mixture of seven neutral amino acids, which resulted in partial recovery of polyribosomes, also tended to reverse the changes in ribosomal protein phosphorylation. Variations in the activities of ribonuclease enzymes in the cerebral cytosol were also observed under these conditions. Administration of phenylalanine increased the activities of cerebral ribonucleases, whereas subsequent treatment with the amino acid mixture partly reversed this effect. The results suggest that alterations in cerebral ribosomal protein phosphorylation, ribosome aggregation and ribosome function are interrelated in experimental hyperphenylalaninaemia.

It is generally assumed that defects in the metabolism of myelin and other brain proteins are responsible for certain of the neurological manifestations in phenylketonuria and other human amino-acidopathies (Menkes, 1968; Prensky *et al.*, 1968; Shah *et al.*, 1972). This concept is consistent with the finding that experimental hyperphenylalaninaemia in immature animals can result in abnormal myelination in the central nervous system and behavioural modifications reminiscent of altera-

tions observed in human phenylketonuria (Gaul *et al.*, 1975). Although several investigators have reported that amino acid incorporation into total brain proteins *in vivo* was acutely decreased in experimental hyperamino-acidaemia, conflicting results have also been obtained (for reviews, see Davison, 1973; Dunlop *et al.*, 1975, 1977; Roberts & Morelos, 1976; Hughes & Johnson, 1978a). Nevertheless, the synthesis of certain brain proteins (e.g. myelin proteins) may be selectively inhibited

under these circumstances (Agrawal *et al.*, 1970). Moreover, chronic hyperamino-acidaemia may uniformly result in a generalized decrease of brain protein synthesis (Prensky *et al.*, 1974).

Several observations with isolated ribosomal preparations *in vitro* support the concept that brain protein synthesis is defective in experimental hyperamino-acidaemia. Thus, parenteral administration of loading doses of phenylalanine or certain other amino acids to immature animals results in acute decreases in the state of aggregation of brain polyribosomes subsequently isolated from these animals (Aoki & Siegel, 1970; MacInnes & Schlesinger, 1971; Siegel *et al.*, 1971; Weiss *et al.*, 1971; Wong *et al.*, 1972; Roberts, 1974; Taub & Johnson, 1975). In addition, brain ribosome preparations from hyperamino-acidaemic animals may exhibit a decreased capacity for amino acid incorporation *in vitro* (Aoki & Siegel, 1970; MacInnes & Schlesinger, 1971; Wong *et al.*, 1972). Evidence has been presented that indicates that alterations in brain protein-synthesizing systems in experimental hyperphenylalaninaemia are associated with defects in initiation processes in brain protein synthesis (Hughes & Johnson, 1978b), as well as the release of lysosomal ribonucleases and other lytic enzymes into the cerebral cytosol (Roberts & Morelos, 1976; Roberts, 1977). The present investigations were undertaken as part of an effort to elucidate the physicochemical basis for alterations in cerebral ribosome structure and function observed in experimental hyperamino-acidaemia. The results indicate that the decreased recovery of cerebral polyribosomes in hyperphenylalaninaemic infant rats is accompanied by profound changes in cerebral ribosomal protein phosphorylation.

Experimental

Materials

Sucrose (Ribonuclease-free) urea and Tris (all ultrapure) were obtained from Schwarz-Mann, Orangeburg, NY, U.S.A. Urea solutions were further purified by passage through a column of Bio-Rad Dowex AG 501 [X8 (D)] resin before use. Schwarz-Mann was also the source of potassium polyuridyate [poly(U)] and [2-¹⁴C]poly(U) (sp. radioactivity 0.44 Ci/mol of polynucleotide phosphorus). [³²P]P_i (carrier-free) in 0.02 M-HCl (285 Ci/mg), obtained from ICN Pharmaceuticals Inc., Irvine, CA, U.S.A., was neutralized with 0.2 M-NaOH and supplemented with NaCl to a final concentration of 0.9% (w/v) NaCl just before administration. Reagents and apparatus for electrophoresis of ribosomal proteins in acrylamide gels were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Sigma was the source of puromycin hydrochloride and β -mercaptoethanol.

L-Amino acids (all A grade) were purchased from Calbiochem, San Diego, CA, U.S.A. Inorganic reagents were analytical-reagent grade from Mallinckrodt, St. Louis, MO, U.S.A., or J. T. Baker, Phillipsburg, NJ, U.S.A. Scintillation-counting solution (3a70B) was obtained from Research Products International Inc., Elk Grove Village, IL, U.S.A. Packard Instrument Co. (La Grange, IL, U.S.A.) supplied PBD [5-(biphenyl-4-yl)-2-phenyl-1-oxa-3,4-diazole] and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene].

Treatment of animals

Infant male rats of an inbred Sprague-Dawley strain, 7–8 days old and weighing 18–20 g, were used in these investigations. The animals were kept warm under an incandescent lamp throughout the experiment.

Each animal was given an intraperitoneal injection of L-phenylalanine in 0.45% (w/v) NaCl (adjusted to pH 7) or an equivalent volume of 0.9% (w/v) NaCl. Phenylalanine was administered either as a 2.5% (w/v) solution (4 ml/100 g body wt.) or a 3.0% (w/v) solution (6.7 ml/100 g body wt.). In some experiments with the higher loading dose of phenylalanine, the animals received a second intraperitoneal injection of 0.9% NaCl or a mixture of seven neutral amino acids 1 h later, in amounts equivalent to 2 ml/100 g body wt. The amino acid mixture contained the L-isomers of leucine, isoleucine, valine, threonine, tyrosine, tryptophan and methionine, each at a concentration of 3.75 mg/ml of acidified 0.9% NaCl (Hughes & Johnson, 1978b). Phosphorylated ribosomal proteins in cerebral cortices of these animals were labelled by intracisternal administration of 2 mCi of [³²P]P_i in 20 μ l of 0.9% NaCl at pH 7. The intracisternal route was selected because only very small amounts of radioactivity were incorporated into brain ribosomal proteins when [³²P]P_i was given intraperitoneally or intravenously. This intracisternal procedure had no discernible effect on patterns of cerebral ribosomal proteins obtained by two-dimensional electrophoresis (Roberts & Morelos, 1979) or on the sedimentation properties of purified cerebral ribosomes (Roberts & Morelos, 1976). The animals were routinely killed by decapitation. However, the results obtained with animals killed under light anaesthesia with sodium pentobarbital were quite similar (Roberts & Morelos, 1976, 1979). The exact dosages and conditions employed in each experiment are described in the Results section.

At the end of each experiment, brains were rapidly removed, placed on filter paper moistened with 0.9% NaCl in a Petri dish kept on ice and immediately transferred to a cold room. Cerebral cortices were then dissected free of underlying tissue, weighed on a torsion balance, washed with buffer to remove

adventitious radioactivity and homogenized as described below.

Preparation of cerebral ribosomes and ribosomal subunits

Several different ribosome preparations were used in these investigations. Cerebral tissue was homogenized in medium (1:3, w/v) containing 0.25 M-sucrose, 12 mM-MgCl₂, 100 mM-KCl, 6 mM- β -mercaptoethanol, 50 mM-Tris/HCl, pH 7.6 at 4°C, and ribonuclease inhibitor protein (50 μ g/ml), as described previously (Roberts & Morelos, 1976). Recovery of cerebral polyribosomes was greater in this medium than in media of lower ionic strength (Zomzely *et al.*, 1966). In some experiments, 40 mM-NaF or 20 mM-sodium molybdate was added to the homogenization medium to inhibit phosphoprotein phosphatase activity (Revel & Racker, 1960; Weller & Rodnight, 1971; Maeno & Greengard, 1972). These additives had no discernible effect on ribosome aggregation or on ribosomal protein phosphorylation (see the Results section). The homogenates were centrifuged at 8000g for 10 min at 0°C to obtain the postmitochondrial supernatant fraction, which was used to prepare cerebral ribosomes. In addition, 0.1 ml samples of the postmitochondrial supernatant preparation were treated with 0.9 ml of 10% (w/v) trichloroacetic acid to precipitate the proteins. After centrifugation, radioactivity in the resulting supernatant fraction was determined as a measure of ³²P uptake into the cerebral cytosol.

Purified cerebral ribosomes were prepared by two general procedures. When the influence of parenteral administration of amino acids on the state of aggregation of cerebral ribosomes was to be assessed by sucrose-density-gradient analysis, postmitochondrial supernatant fractions pooled from three to six animals in each group were centrifuged in a Spinco 50 Ti rotor for 4 or 20 h at 226 400g and 0°C through 2 M-sucrose that contained 12 mM-MgCl₂, 100 mM-KCl, 6 mM- β -mercaptoethanol, 50 mM-Tris/HCl, pH 7.6 at 4°C, and ribonuclease-inhibitor protein (50 μ g/ml). The resulting pellets were resuspended in this medium and layered on to 10–40% (w/v) linear sucrose gradients containing the same medium in a Spinco SW 50.1 swinging-bucket rotor. After centrifugation at 185 000g and 0°C for 60 min, the gradients were analysed for material that absorbed u.v. light at 254 nm by the use of an ISCO model 640 density-gradient fractionator. These ribosome preparations consisted of various proportions of monoribosomes and polyribosomes in animals subjected to different treatments. A scaled-up version of this procedure was used to obtain separate populations of cerebral monoribosomes and polyribosomes for studies of ribosomal protein phosphorylation. Postmito-

chondrial supernatant fractions were pooled from 24–48 animals and centrifuged in a Spinco 70 Ti rotor for 4 or 20 h at 312 000g and 0°C through the 2 M-sucrose medium described above. In some cases, this medium also contained 40 mM-NaF or 20 mM-sodium molybdate. The pelleted ribosomes were resuspended in the homogenization medium, layered on to 15–35% linear sucrose gradients containing the same other additives as the 2 M-sucrose medium, and centrifuged in a Spinco SW 27 swinging-bucket rotor at 131 000g and 0°C for 2.75 h. Effluents from the tops of the gradients were collected as 0.6 ml fractions. Appropriate fractions were combined to give separate monoribosome and polyribosome populations. The monoribosome fraction also contained dimeric ribosomes that form spontaneously in rat brain preparations in the presence of 4–12 mM-Mg²⁺ (Zomzely *et al.*, 1966). The separated monoribosome and polyribosome fractions were pelleted by centrifugation for 20 h at 226 400g and 0°C in a Spinco 50 Ti rotor. These fractions were dissociated into subunits as described below.

A modified procedure was employed in studies of ribosomal protein phosphorylation to prepare purified ribosomes that were to be analysed without further subdivision into monoribosome and polyribosome fractions. These ribosomes were isolated as described above except that the 2 M-sucrose medium contained 500 mM-KCl. Just before dissociation into subunits, this ribosome preparation was suspended in medium that contained 0.25 M-sucrose, 5 mM-MgCl₂, 500 mM-KCl, 6 mM- β -mercaptoethanol, 50 mM-Tris/HCl, pH 7.6 at 4°C, and ribonuclease-inhibitor protein (50 μ g/ml). This suspension was then centrifuged at 226 400g and 0°C for 3.5 h to pellet the ribosomes. The high concentrations of KCl used in these purification steps effectively removes proteins that are not part of the ribosome structure (Warner & Pène, 1966; Sherton & Wool, 1974; Ashby & Roberts, 1975). Certain of these proteins are actively phosphorylated in rat cerebral cortex *in vivo* and *in vitro* (Ashby & Roberts, 1975; Holbrook & Roberts, 1979). Since exposure to 500 mM-KCl also resulted in some disaggregation and dissociation of cerebral polyribosomes (see the Discussion section), this high-salt treatment could not be used when separate monoribosome and polyribosome fractions were to be isolated. However, subsequent treatment of these separated fractions with high concentrations of KCl was carried out. Thus, ribosomal subunits were prepared from the various ribosome preparations by incubation at 37°C for 20 min in the presence of 0.2 mM-puromycin, 800 mM-KCl, 12.5 mM-MgCl₂, 20 mM- β -mercaptoethanol and 50 mM-Tris/HCl, pH 7.5 at 22°C (Leader & Wool, 1972). The subunits were then separated by centrifugation of this suspension in a Spinco SW 27 swinging-bucket

rotor for 17.5 h at 131000 *g* and 20°C on a linear 10–30% (w/v) sucrose gradient containing 5 mM-MgCl₂, 580 mM-KCl, 20 mM- β -mercaptoethanol and 10 mM-Tris/HCl, pH 7.5 at 22°C (Roberts & Ashby, 1978). The cerebral subunits were separately isolated by collecting those fractions of the 40S and 60S bands, which exhibited absorbances above the half-heights of the peaks on the density-gradient recordings. The pooled fractions of each subunit were diluted to give a final concentration of 3 mM-MgCl₂ and pelleted by centrifugation for 20 h at 226400 *g* and 0°C in a Spinco 50 Ti rotor. The 60S subunits were re-dissociated and re-fractionated as described above to remove contaminating 80S ribosomes and 40S subunits. Cerebral 40S and 60S subunits isolated by this procedure appeared to be virtually free of cross-contamination and non-ribosomal phosphorylated proteins (Roberts & Ashby, 1978; Roberts & Morelos, 1979).

Two-dimensional electrophoresis of cerebral 40S ribosomal proteins

Ribosomal proteins were isolated from the purified 40S and 60S subunits essentially as described previously (Barritault *et al.*, 1976; Roberts & Ashby, 1978). The proteins were dissolved with frequent stirring with a small spatula for 6 h in 150 μ l of medium composed of 8 M-urea and 10 mM- β -mercaptoethanol. Samples (10 μ l) were taken in duplicate for measurement of protein and radioactivity. In addition, a measured volume of the 40S ribosomal protein preparation (80–120 μ l) was subjected to two-dimensional electrophoresis by a modification of the procedure of Howard & Traut (1974) as outlined by Roberts & Ashby (1978). The first-dimensional cylindrical gel contained 8% acrylamide and 6 M-urea buffered at pH 8.6 and was maintained at 23°C during electrophoresis. The proteins were applied to the anode and stacked for 1 h with a current of 3 mA/gel (100 V). Electrophoresis was then allowed to proceed toward the cathode at 3–4 mA/gel (100–150 V) for 14–18 h. Electrophoresis in the second dimension was carried out in slab gels containing 18% acrylamide and 6 M-urea buffered at pH 4.5. The ribosomal proteins were stacked at the anode for 1 h at 16 mA/gel (40 V) and then subjected to electrophoresis toward the cathode for 4.5–5.0 h at approx. 100 mA/gel (300 V). The slab-gel apparatus was cooled by circulating refrigerant kept at 0°C. The slabs were stained with Coomassie Brilliant Blue for 3 h and then destained by diffusion overnight. In some instances, the gel slabs were subsequently dried and subjected to autoradiography against Kodak No-Screen X-ray film. In other cases, the region of the gel slabs corresponding to the S6 protein of the cerebral 40S subunit was excised, scanned at 600 nm and then cut into 1-mm sections for scintillation counting of

radioactivity after solubilization in 30% H₂O₂ (Ashby & Roberts, 1975).

Measurement of ribonuclease activities in the cerebral cytosol

Modifications of the sensitive method of Barondes & Nirenberg (1962), which monitors the degradation of poly(U), were used to measure ribonuclease activities in the cerebral cytosol (Zomzely *et al.*, 1968; Szego *et al.*, 1971, 1974; Roberts & Morelos, 1976). These modifications were designed to measure ribonuclease activity in cerebral postmitochondrial supernatant fractions prepared and assayed in the usual homogenization medium with liver ribonuclease inhibitor omitted, as well as to determine enzyme activity under conditions that were selective for acid ribonuclease II of lysosomal origin (Razzell, 1967; Barnard, 1969). In the latter instances, cerebral cortices were homogenized in 0.25 M-sucrose and the postmitochondrial supernatant fractions were prepared and diluted with an equal volume of 0.1 M-sodium acetate in 0.25 M-sucrose at pH 5.0 for incubation with [¹⁴C]poly(U). Triplicate samples were taken for assay, each representing 25 mg of tissue. The assay medium contained 0.25 μ mol (2 m μ Ci) of [¹⁴C]poly(U) as polynucleotide phosphorus in a final volume of 0.2 ml of the appropriate medium. Incubation was carried out in air at 37°C for periods varying from 10 to 60 min. Undegraded poly(U) was precipitated by addition of a mixture of 2 vol. of absolute ethanol and 1 vol. of 0.1 M-magnesium acetate. The samples were kept at 0°C for 30 min and then 2 ml of cold 0.05 M-magnesium acetate in 50% (v/v) ethanol was added. The precipitates were collected on Millipore filters, washed repeatedly with the latter solution and analysed for radioactivity. The rate of degradation of poly(U) by cerebral ribonucleases under these conditions was linear for 60 min of incubation (Roberts & Morelos, 1976).

Radioactivity measurements

³²P radioactivity was measured in the cerebral cytosol and in the purified 40S and 60S ribosomal proteins as described previously (Roberts & Morelos, 1979). Millipore filters from the ribonuclease assays were assayed for ¹⁴C radioactivity in 10 ml of POPOP/PBD scintillation solution. Efficiency of detection of ³²P and ¹⁴C radioactivity in a Packard Tri-Carb scintillation spectrometer exceeded 90 and 50% respectively.

Protein analyses

Analyses of protein content of the postmitochondrial supernatant preparations and the various ribosome fractions were carried out by the method of Lowry *et al.* (1951) with the use of bovine serum albumin as a standard.

Results

Effect of phenylalanine loading on kinetics of phosphorylation of cerebral ribosomal proteins

Incorporation of radioactivity from intracisternally administered [^{32}P]P_i into proteins of cerebral cortical ribosomes continued actively for at least 2 h in saline-treated infant rats (Table 1). The phosphorylated proteins were presumably constitutive ribosomal proteins, since they satisfied the criterion that they were not removed by repeated exposure of the ribosomes to high concentrations of KCl (Warner & Pène, 1966; Sherton & Wool, 1974). The phosphorylation process involved principally esterification of serine hydroxy groups of the cerebral ribosomal proteins (Ashby & Roberts, 1975; Roberts & Morelos, 1979).

A different pattern of ribosomal protein phosphorylation was observed in cerebral cortices of similar animals given a moderate excess of L-phenylalanine (1 mg/g body wt.) intraperitoneally, instead of saline. ^{32}P -labelling of the cerebral 40S ribosomal proteins was significantly decreased below control values within 30 min of phenylalanine administration (Table 1). In four experiments, the decrease in ^{32}P incorporation into 40S ribosomal proteins averaged $27 \pm 2\%$ at 30 min after administration of L-phenylalanine, $39 \pm 3\%$ after 1 h, and $55 \pm 5\%$ after 2 h. Only minor changes in phosphorylation of 60S ribosomal proteins were observed over this period. The changes in phosphorylation of 40S ribosomal proteins after phenylalanine treatment were not associated with sig-

nificant variations in uptake of intracisternally administered ^{32}P into the cerebral cytosol (Table 1).

The apparent inhibitory effect of phenylalanine loading on phosphorylation of 40S ribosomal proteins in cerebral cortices of infant rats disappeared after about 3 h. Thus, when [^{32}P]P_i was given 2 h after the intraperitoneal administration of phenylalanine, and the animals were killed 1 h later, the specific radioactivity of the cerebral 40S ribosomal proteins averaged 94.3 c.p.m./ μg in two experiments. The analogous value for saline-treated rats was 109.9 c.p.m./ μg of ribosomal protein.

Previous investigations have shown that the moderate excess of L-phenylalanine employed in this set of experiments (1 mg/g body wt.) resulted in a maximal increase in plasma phenylalanine values in infant rats within about 30 min of administration by the intraperitoneal route (Siegel *et al.*, 1971; B. H. Bogucki & S. Roberts, unpublished work). The increase in plasma phenylalanine content was comparable with that observed in human phenylketonuria (Scriver & Rosenberg, 1973), and was accompanied by a 20-fold increase in the cerebral concentration of phenylalanine (Roberts & Morelos, 1976). In the present studies, ribosomal disaggregation in purified ribosome preparations from infant rat cerebral cortex, isolated by centrifugation through heavy sucrose for 20 h, was also maximal about 30 min after intraperitoneal administration of the same dose of phenylalanine (Fig. 1). Complete recovery of polyribosomes subsequent to the administration of this loading dose of phenylalanine required about 4 h (see also Siegel *et al.*, 1971).

Table 1. *Influence of phenylalanine loading on kinetics of uptake of [^{32}P]P_i into the cerebral cytosol and phosphorylation of proteins in ribosomal subunits of rat cerebral cortex in vivo*

Infant rats were injected intraperitoneally with 2.5% L-phenylalanine (1 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. Immediately thereafter, 2 mCi of [^{32}P]P_i was administered intracisternally to each animal in 20 μl of 0.9% NaCl. The rats were killed at the intervals indicated. Uptake of ^{32}P into the cerebral cytosol was estimated from the amount of radioactivity in the trichloroacetic acid-soluble material of the post-mitochondrial supernatant fractions from which the ribosomes were isolated. Samples of the purified ribosomal proteins were analysed for protein and radioactivity. Specific radioactivities were corrected for radioactive decay over the period between administration of isotope and measurement of radioactivity. Each analysis represents the cytosol or ribosomes pooled from eight animals. The results shown are means $\pm$ S.E.M. of four separate experiments.

Time after injection (min)	$10^{-5} \times ^{32}\text{P}$ uptake (c.p.m./g of cortex)	Radioactivity in ribosomal proteins (c.p.m./ μg)	
		40S	60S
Saline-injected			
30	2188 ± 161	63.7 ± 5.3	6.0 ± 0.9
60	2922 ± 140	90.4 ± 6.3	9.1 ± 1.2
120	4305 ± 149	137.4 ± 8.4	11.3 ± 1.5
Phenylalanine-injected			
30	2459 ± 184	46.2 ± 4.5	6.8 ± 1.0
60	3196 ± 229	56.0 ± 5.5	8.3 ± 0.8
120	4353 ± 270	59.0 ± 6.6	9.5 ± 0.9

Fig. 1. Influence of a moderate excess of phenylalanine on the state of aggregation of purified polyribosomes from cerebral cortices of infant rats

Each animal was injected intraperitoneally with 2.5% L-phenylalanine (1 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. The rats were killed at intervals of 30 min to 4 h and the ribosomes were isolated from the pooled cerebral cortices of three animals in each group by centrifugation for 20 h through medium containing 2 M-sucrose, as described in the text. Sucrose-density-gradient analyses of the purified ribosomes were carried out. The monoribosome peaks are indicated as 80S. (a) Animals given saline 30 min earlier; (b) animals given phenylalanine 30 min earlier; (c) animals given phenylalanine 1 h earlier; (d) animals given phenylalanine 2 h earlier; (e) animals given phenylalanine 3 h earlier; (f) animals given phenylalanine 4 h earlier.

Saline treatment had no effect on the polyribosome profile at any time interval. Thus the phenylalanine-induced variations in ribosomal protein phosphorylation in rat cerebral cortex *in vivo* appeared to follow a progression that was somewhat similar to the time course of polyribosome disappearance and reappearance in isolated ribosome preparations *in vitro*. Alterations in the state of aggregation of cerebral ribosomes in analogous preparations obtained by centrifugation through 2 M-sucrose for only 4 h, instead of 20 h, were comparable with those depicted in Fig. 1 (see for example, Roberts & Morelos, 1976). Since the longer period of centrifugation enhanced the recovery of cerebral monoribosomes and polyribosomes about 20%, this procedure was usually employed in the present investigations to provide purified ribosomes for studies of ribosomal protein phosphorylation. However, these variations in centrifugation time had no discernible effect on the phosphorylation state of cerebral ribosomal proteins (see below).

Effects of phenylalanine loading on phosphorylation of the S6 protein of cerebral 40S ribosomal subunits

Although several constitutive proteins of both ribosomal subunits are capable of incorporating

radioactivity from [32 P] P_i in rat cerebral cortex *in vivo*, 80–90% of this radioactivity is associated with the S6 protein of the 40S subunit (Roberts & Morelos, 1979). This cerebral protein normally exists in several states of phosphorylation that can be detected by two-dimensional electrophoresis on polyacrylamide gels containing urea (Fig. 2a). Administration of a moderate excess of L-phenylalanine (1 mg/g body wt.) intraperitoneally to infant rats decreased the proportions of the more highly phosphorylated derivatives of the cerebral S6 protein, which travelled more slowly in the first dimension at pH 8.6 than the less phosphorylated species. Thus, 1 h after phenylalanine treatment, the more acidic components of this protein were markedly depleted on the Coomassie Blue-stained electrophoretograms (Fig. 2b). The control pattern of phosphorylation of the cerebral S6 ribosomal protein was re-established within about 3 h of intraperitoneal administration of this dose of phenylalanine (Fig. 2c).

Autoradiographs of the two-dimensional electrophoretograms indicated that the proportion of radioactivity from intracisternally administered P_i incorporated into the more highly phosphorylated derivatives of the cerebral S6 protein was decreased 1 h after phenylalanine treatment (Fig. 2e), com-

Fig. 2. Influence of a moderate excess of phenylalanine on two-dimensional electrophoretograms of proteins isolated from the 40S ribosomal subunit of rat cerebral cortex

Infant rats were injected intraperitoneally with 2.5% L-phenylalanine (1 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. Either immediately thereafter, or 2 h later, the animals received 2 mCi of [32 P]P_i intracisternally in 20 μ l of 0.9% NaCl. All animals were killed 1 h after the last injection. Cerebral ribosomes were isolated in the presence of 40 mM-NaF. Proteins extracted from the small cerebral subunit were subjected to two-dimensional electrophoresis on polyacrylamide gels containing urea. (a), (b) and (c) show electrophoretograms stained for protein with Coomassie Brilliant Blue; (d), (e) and (f) show autoradiographs exposed for 1 day. The amounts of ribosomal protein applied to the first-dimensional gels were: (a) and (d), 429 μ g; (b) and (e), 409 μ g; (c) and (f), 433 μ g. In (a) and (d), rats were given [32 P]P_i immediately after saline; in (b) and (e), rats were given [32 P]P_i immediately after phenylalanine; in (c) and (f), rats were given [32 P]P_i 2 h after phenylalanine. The position of S6 protein is marked on the electrophoretograms.

pared with the corresponding process in cerebral 40S subunits from saline-treated rats (Fig. 2d). When the [32 P]P_i was administered 2 h after phenylalanine, and the rats were killed 1 h later, the distribution of radioactivity among the congeners of the cerebral S6 protein was not noticeably different from that observed in animals given saline (Fig. 2f).

Effects of a mixture of seven neutral amino acids on phenylalanine-induced alterations in ribosomal protein phosphorylation in rat cerebral cortex

Hughes & Johnson (1977a, 1978b) reported that administration of a mixture of seven neutral amino acids intraperitoneally partly reversed the disaggregated state of brain polyribosomes induced in young mice by injection of a large excess of phenylalanine. In the present investigations, this phenomenon was reproduced in infant rats (Fig. 3). The amount of phenylalanine administered intraperitoneally (2 mg/g body wt.) was twice the load used in the preceding experiments. In contrast with the results with the smaller load (Fig. 1), the maximal degree of cerebral polyribosome disaggregation was observed 1 h (Fig. 3c), rather than 30 min (Fig. 3b), after administration of the higher dose of phenylalanine. Moreover, disaggregation was much more extensive. Utilization of the higher loading dose of phenylalanine

permitted clear-cut demonstration of partial recovery of cerebral polyribosomes when the amino acid mixture was administered 1 h after phenylalanine loading, and the animals were killed after one additional hour (cf. Figs. 3e and 3f). Administration of the amino acid mixture 1 h after saline treatment resulted in disaggregation of cerebral polyribosomes (results not shown). The amino acid mixture contained the seven neutral amino acids, which were markedly decreased in the cerebrum of infant rats given a loading dose of phenylalanine intraperitoneally (Roberts & Morelos, 1976).

In common with the results of studies with the smaller loading dose of phenylalanine, intraperitoneal administration of this amino acid to infant rats in amounts equivalent to 2 mg/g body wt. resulted in a marked decrease in incorporation of intracisternally administered [32 P]P_i into cerebral 40S ribosomal proteins (Table 2). Uptake of 32 P into the cerebral cytosol and incorporation of radioactivity into ribosomal proteins of the 60S subunit exhibited little change under these circumstances. Decreased incorporation of radioactivity into cerebral 40S ribosomal proteins in the presence of the larger load of phenylalanine, as well as the smaller load, was accompanied by depletion of the more highly phosphorylated species of the S6 protein. This

Fig. 3. Reversal of phenylalanine-induced disaggregation of cerebral polyribosomes by a mixture of seven neutral amino acids

Infant rats were each injected intraperitoneally with 3.0% L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. Some animals from each of these two groups were killed after 30 or 60 min. At the later time interval, the remaining animals in each group received either a mixture of seven neutral amino acids (2 ml/100 g body wt.), or an equivalent volume of 0.9% NaCl, by the same route. The mixture of amino acids was composed of the L-isomers of leucine, isoleucine, valine, threonine, tyrosine, tryptophan and methionine, each present at a concentration of 3.75 mg/ml of 0.9% NaCl. These animals were killed 1 h after the second injection. Ribosomes were isolated from the pooled cerebral cortices of three animals in each group by centrifugation for 20 h through medium containing 2 M-sucrose. Sucrose-density-gradient analyses of the purified polyribosomes were carried out. The monoribosome peaks are indicated as 80S. (a) Animals given saline 1 h earlier; (b) animals given phenylalanine 30 min earlier; (c) animals given phenylalanine 1 h earlier; (d) animals given saline followed 1 h later by a second injection of saline and then killed after an additional hour; (e) animals given phenylalanine followed by saline 1 h later; (f) animals given phenylalanine followed by the amino acid mixture 1 h later.

Table 2. Influence of a mixture of seven neutral amino acids on phenylalanine-induced alterations in the incorporation of radioactivity from [32 P] P_i into cerebral ribosomal proteins

Infant rats were injected intraperitoneally with 3.0% L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. After 45 min, each animal received 2 mCi of [32 P] P_i intracisternally in 20 μ l of 0.9% NaCl. The second intraperitoneal injection of the amino acid mixture or 0.9% NaCl was given 15 min thereafter in amounts equivalent to 2 ml/100 g body wt. The rats were killed 1 h after the last injection. Postmitochondrial supernatant fractions from six rats in each group of 18 animals were combined to give three homologous pools before sampling for measurements of 32 P uptake. The remainder of these fractions from similarly treated animals were then combined for ribosome isolation. The results shown are means $\pm$ S.E.M. of three separate experiments. See the legend to Table 1 for further explanation.

Intraperitoneal injections		$10^{-5} \times ^{32}\text{P}$ uptake (c.p.m./g of cortex)	Radioactivity in ribosomal proteins (c.p.m./ μ g)	
First	Second		40S	60S
Saline	Saline	3331 $\pm$ 371	124.9 $\pm$ 8.9	13.0 $\pm$ 1.2
Phenylalanine	Saline	2522 $\pm$ 236	46.0 $\pm$ 3.2	10.8 $\pm$ 1.1
Phenylalanine	Amino-acid mixture*	2846 $\pm$ 374	86.3 $\pm$ 5.9	11.4 $\pm$ 1.2

* A mixture of leucine, isoleucine, valine, threonine, tyrosine, tryptophan and methionine, each present at a concentration of 3.75 mg/ml of 0.9% NaCl.

phenomenon was clearly demonstrated when the region corresponding to the elongated S6 protein on two-dimensional gels was excised, scanned for absorbance at 600nm then cut into 1mm wide sections for determination of radioactivity. The radioactivity in the S6 region was associated with four or five protein species on the gels that stained with Coomassie Brilliant Blue (Fig. 4a). However, the stained portion of the cerebral S6 ribosomal protein with the greatest electrophoretic mobility in the first dimension was not in itself radioactive. Administration of L-phenylalanine intraperitoneally not only decreased the relative absorbances of the phosphorylated congeners of the S6 protein compared with the unlabelled non-phosphorylated species, but also decreased the total and relative amounts of radioactivity incorporated into the more highly phosphorylated components (Fig. 4b). Subsequent administration of a mixture of seven neutral amino acids, which resulted in partial recovery of cerebral polyribosomes, also tended to restore the more highly phosphorylated congeners of the S6 protein (Fig. 4c) and increased the labelling of the cerebral 40S proteins (Table 2).

Effects of phenylalanine loading and subsequent administration of a mixture of seven neutral amino acids on phosphorylation of proteins of the 40S subunit in separated cerebral monoribosomes and polyribosomes

Evidence has been presented that phosphorylation of the S6 ribosomal protein in eukaryotic cells is either normally restricted to polyribosomes or is a much more active process in polyribosomes than monoribosomes (Bitte & Kabat, 1972; Leader & Coia, 1978). This phenomenon was clearly evidenced in the present investigations when purified ribosomes from cerebral cortices of saline-injected infant rats were fractionated into monoribosomes and polyribosomes. Ribosomal proteins isolated from 40S subunits of the separated polyribosome fraction incorporated five to ten times as much radioactivity from intracisternally administered [^{32}P]P_i as the analogous preparations from the monoribosome fraction (Table 3). This relationship was strikingly altered by the intraperitoneal administration of an excess of L-phenylalanine, in either the moderate dose (Expt. 1) or the high dose (Expt. 2). Under these circumstances, the specific radioactivities of 40S ribosomal proteins from separated polyribosome and monoribosome populations tended to approach equality, as a consequence of both decreased labelling of polyribosomal proteins and increased labelling of monoribosomal proteins. Administration of a mixture of seven neutral amino acids, 1h after the injection of the higher dose of phenylalanine, partly restored the labelling pattern of

Fig. 4. Reversal of phenylalanine-induced alterations in phosphorylation of the cerebral S6 ribosomal protein by a mixture of seven neutral amino acids

Infant rats were each injected intraperitoneally with 3.0% L-phenylalanine (2mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. After 45 min, the animals received 2mCi of [^{32}P]P_i intracisternally in 20 μ l of 0.9% NaCl. After an additional interval of 15 min, the animals given saline received 0.9% NaCl, whereas the animals given phenylalanine were injected intraperitoneally with either 0.9% NaCl or a mixture of seven neutral amino acids as outlined in the legend to Fig. 3. The animals were killed 1h after the second intraperitoneal injection. Cerebral ribosomes were isolated by centrifugation for 4h through medium containing 2M-sucrose. Proteins extracted from the small cerebral subunit were subjected to two-dimensional electrophoresis. The amounts of protein applied to the first-dimensional gels were: (a) 523 μ g; (b) 506 μ g; (c) 512 μ g. After the two-dimensional slab gels were stained with Coomassie Brilliant Blue, a horizontal rectangular section corresponding to the S6 protein and its more acidic phosphorylated derivatives was cut out and scanned for absorbance at 600nm (—). This section of the gel was then divided into 1-mm slices for determination of radioactivity (●—●—●). (a) Animals given saline followed by a second injection of saline 1h later; (b) animals given phenylalanine followed by saline 1h later; (c) animals given phenylalanine followed by the amino acid mixture 1h later.

Table 3. *Influence of phenylalanine loading and subsequent administration of a mixture of seven neutral amino acids on differential phosphorylation of proteins of the 40S subunit in cerebral monoribosomes and polyribosomes*

Infant rats were each given 2 mCi of [32 P]P_i intracisternally in 20 μ l of 0.9% NaCl. Each group was composed of 24 rats. In Expt. 1, the isotope was given at zero time; 0.9% NaCl or 2.5% L-phenylalanine was administered intraperitoneally immediately thereafter. In Expt. 2, the isotope was given 45 min after the first intraperitoneal injection of 0.9% NaCl or 3.0% L-phenylalanine. The second intraperitoneal injection of 0.9% NaCl or the mixture of seven neutral amino acids was administered 15 min after the isotope. In all instances the rats were killed 1 h after the last injection. The results are means $\pm$ S.E.M. of three separate experiments. See the legends to Tables 1 and 2 for further explanation.

Intraperitoneal injections		Radioactivity in 40S ribosomal proteins (c.p.m./ μ g)	
First	Second	Monoribosomes	Polyribosomes
Expt. 1			
Saline	—	17.8 $\pm$ 1.5	128.0 $\pm$ 12.9
Phenylalanine (1 mg/g body wt.)	—	77.6 $\pm$ 5.9	66.4 $\pm$ 6.2
Expt. 2			
Saline	Saline	18.8 $\pm$ 2.3	145.4 $\pm$ 9.4
Phenylalanine (2 mg/g body wt.)	Saline	47.4 $\pm$ 4.6	38.6 $\pm$ 3.8
Phenylalanine (2 mg/g body wt.)	Amino acid mixture	26.4 $\pm$ 3.4	99.9 $\pm$ 10.1

Fig. 5. *Influence of a large excess of phenylalanine and a mixture of seven neutral amino acids on two-dimensional electrophoretograms of proteins isolated from the 40S ribosomal subunit of separated cerebral polyribosomes (a, b and c) and monoribosomes (d, e and f)*

Infant rats were injected intraperitoneally with 3.0% L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. After 45 min, the animals were each given 2 mCi of [32 P]P_i intracisternally in 20 μ l of 0.9% NaCl. After an additional 15 min, the animals were injected intraperitoneally with either 0.9% NaCl or a mixture of seven neutral amino acids as described in the legend to Fig. 3. The animals were killed 1 h after the second intraperitoneal injection. Purified cerebral ribosomes were prepared and fractionated into polyribosomes and monoribosomes as described in the text. Proteins extracted from the small subunit were subjected to two-dimensional electrophoresis. The amounts of protein applied to the first-dimensional gels were (in μ g): (a) 421; (b) 420; (c) 412; (d) 406; (e) 398; (f) 402. (a) and (d), animals given saline followed by a second injection of saline 1 h later; (b) and (e), animals given phenylalanine followed by saline 1 h later; (c) and (f), animals given phenylalanine followed by the amino acid mixture 1 h later. The position of the S6 protein is marked on the electrophoretograms.

cerebral monoribosomes and polyribosomes observed in control animals.

These differences in overall labelling of the 40S ribosomal proteins in cerebral monoribosome and polyribosome fractions could be ascribed principally to variations in phosphorylation of the S6 protein. Thus two-dimensional gel electrophoresis of the 40S ribosomal proteins revealed that separated cerebral polyribosomes from saline-treated infant rats contained relatively large proportions of the more highly phosphorylated congeners of the S6 protein that stained with Coomassie Brilliant Blue (Fig. 5a). In contrast, the S6 protein was poorly

phosphorylated in the corresponding cerebral monoribosomes (Fig. 5d). Phenylalanine administration markedly decreased phosphorylation of the S6 protein in cerebral polyribosomes and resulted in monoribosome and polyribosome populations, which contained the S6 protein in essentially equivalent states of phosphorylation (Fig. 5b and 5e). Subsequent treatment with the amino acid mixture tended to restore the control patterns of phosphorylation of the S6 protein (Fig. 5c and 5f). Alterations in the incorporation of ^{32}P into each of the phosphorylated congeners of the cerebral S6 protein under these circumstances were determined

Fig. 6. Reversal of phenylalanine-induced alterations in phosphorylation of the S6 protein in separated cerebral polyribosomes (a, b and c) and monoribosomes (d, e and f) by a mixture of seven neutral amino acids

Infant rats were injected intraperitoneally with 3.0% L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. After 45 min, the animals were each given 2 mCi of [^{32}P]P_i intracisternally in 20 μl of 0.9% NaCl. After an additional 15 min, the animals were injected intraperitoneally with either 0.9% NaCl or a mixture of seven neutral amino acids as described in the legend to Fig. 3. The animals were killed 1 h after the second intraperitoneal injection. Purified cerebral ribosomes were prepared in the presence of 20 mM-sodium molybdate and fractionated into polyribosomes and monoribosomes. Proteins extracted from the small cerebral subunit were subjected to two-dimensional electrophoresis. The amounts of protein applied to the first-dimensional gels were (in μg): (a) 396; (b) 402; (c) 415; (d) 399; (e) 422; (f) 406. After the two-dimensional gels were stained with Coomassie Brilliant Blue, a horizontal rectangular section corresponding to the S6 protein and its more acidic phosphorylated derivatives was cut out and scanned for absorbance at 600 nm (—). This section of the gel was then divided into 1-mm slices for determination of radioactivity (●—●—●). (a) and (d), animals given saline followed by a second injection of saline 1 h later; (b) and (e), animals given phenylalanine followed by saline 1 h later; (c) and (f), animals given phenylalanine followed by the amino acid mixture 1 h later.

Fig. 7. Influence of phenylalanine loading and subsequent administration of a mixture of seven neutral amino acids on ribonuclease activities in cerebral cytosol of infant rats

Each animal was injected intraperitoneally with 3.0% L-phenylalanine (2mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. After 1 h, the animals were injected intraperitoneally with either 0.9% NaCl or a mixture of seven neutral amino acids as described in the legend to Table 2. The animals were killed 2 h after the first injection. Cerebral cortices were pooled from three rats in each group. Postmitochondrial supernatant fluids were assayed for ribonuclease activity in either (a) the usual homogenization medium composed of 0.25M-sucrose, 12mM-MgCl₂, 100mM-KCl and 50mM-Tris/HCl buffer at pH 7.6, or (b) medium composed of 0.25M-sucrose and 0.05M-sodium acetate buffer at pH 5.0 (acid ribonuclease). The values shown are means $\pm$ S.E.M. (where indicated by bars) for triplicate determinations in representative experiments. Symbols: ●, rats given saline followed by a second injection of saline 1 h later; ○, rats given phenylalanine followed by saline 1 h later; △, rats given phenylalanine followed by the amino acid mixture 1 h later.

from scans of the absorbances of the S6 regions excised from two-dimensional gels coupled with measurements of radioactivity present in 1-mm slices of the excised regions. In control animals, the more highly phosphorylated congeners of the cerebral S6 protein were more heavily labelled in the polyribosomes than in monoribosomes (Fig. 6a versus 6d). In animals given the loading dose of phenylalanine, the labelling patterns of cerebral S6 congeners were quite similar in polyribosomes and monoribosomes, as a result of the decrease in phosphorylation of the polyribosomes (Fig. 6b) and the increase in labelling of the monoribosomes (Fig. 6e). Additional treatment of these animals with a mixture of seven neutral amino acids tended to counteract the effects of phenylalanine alone on labelling of the S6 protein in cerebral polyribosomes and monoribosomes (Fig. 6c and 6f).

Effects of phenylalanine loading and subsequent administration of a mixture of seven neutral amino acids on ribonuclease activities in cerebral cytosol of infant rats

The experiments described above indicated that phenylalanine loading in infant rats resulted in the accumulation of cerebral monoribosomes with the

S6 ribosomal protein in a phosphorylation state that closely resembled the altered state of phosphorylation of the S6 protein in the polyribosome population under these conditions. A partial explanation for this phenomenon was suggested by previous findings from this laboratory that revealed that the phenylalanine-induced disaggregation of cerebral polyribosomes in infant rats resulted in a selective loss of the larger polyribosomes and was accompanied by enhanced activity of ribonucleases in the cerebral cytosol (Roberts, 1974; Roberts & Morelos, 1976). In the present studies, administration of a larger loading dose of phenylalanine (2mg/g body wt.) increased ribonuclease activities in the cerebral cytosol about 30% in 2 h (Fig. 7). The increased activity was noted when cerebral ribonuclease was assayed at pH 7.4 in the medium routinely used for the isolation of polyribosomes (Fig. 7a), as well as at pH 5.0 in the absence of added Mg²⁺ (Fig. 7b). The latter assay conditions favour the activity of lysosomal acid ribonuclease (Razzell, 1967; Barnard, 1969). Comparable increases in cerebral ribonuclease activities were also observed 1 h after administration of this dose of phenylalanine (results not shown). Administration of the mixture of seven neutral amino acids 1 h after phenylalanine treatment partly reversed the phenyl-

alanine-induced increase in ribonuclease activities observed at 2 h (Fig. 7).

Discussion

Previous investigations in this laboratory suggested that phenylalanine-induced disaggregation of cerebral polyribosomes in infant rats occurred in at least two distinct phases (Roberts & Morelos, 1976). One phase of this process took place *in vitro* during ribosome isolation and was presumably due to the action of ribonucleases released into the cerebral cytosol by phenylalanine loading (see also Roberts, 1977). Polyribosome disaggregation under these conditions could be partly prevented by addition of native ribonuclease inhibitor to the homogenization and isolation media. An additional component of the disaggregation phenomenon was attributed to processes instituted *in vivo*. Thus, experiments in which cerebral ribosomal RNA in phenylalanine-treated and control animals was differentially labelled *in vivo*, and ribosomes were isolated from pooled cerebral cortices of both groups, revealed that cerebral ribosomes from the hyperamino-acidaemic animals were less highly aggregated than cerebral ribosomes from the control group. However, it was not clear from these results whether polyribosome disaggregation occurred *in vivo* or the polyribosomes were simply sensitized *in vivo* to subsequent disaggregation during isolation. The mechanisms underlying this effect of phenylalanine loading on polyribosome stability *in vivo* also remain to be elucidated. Suggested mechanisms include the action of lysosomal ribonucleases or other lytic enzymes on cerebral polyribosomes *in vivo*, resulting in complexed monoribosomes unable to form initiation complexes (Roberts, 1977), or a primary defect in the initiation of protein synthesis; e.g. due to a deficiency of initiator methionyl-tRNA (Hughes & Johnson, 1977b, 1978b). Although it is not possible to distinguish unequivocally between these two possibilities by inspection of polyribosome profiles, it is clearly evident in the double-label experiments outlined above that the larger cerebral polyribosomes were selectively disaggregated in phenylalanine-treated rats (Roberts & Morelos, 1976). This phenomenon suggests an initial lytic action rather than a primary defect in initiation. Moreover, it is difficult to visualize how a primary defect in initiation processes in itself could result in the alterations in cerebral ribosomal protein phosphorylation observed in hyperphenylalaninaemic animals in the present studies; i.e., enhanced phosphorylation of the S6 protein in cerebral monoribosomes, coupled with decreased phosphorylation of this protein in the corresponding polyribosomes. Thus, the S6 protein in cerebral monoribosomes from untreated animals, as well as

comparable preparations from other eukaryotic cells (Bitte & Kabat, 1972; Leader & Coia, 1978), normally exists in a relatively dephosphorylated state. Phosphorylation of this protein presumably occurs during or after polyribosome formation. Evidence from other studies indicates that a primary block in initiation results in decreased phosphorylation of the S6 ribosomal protein in both messenger-free and complexed ribosomes (Martini & Kruppa, 1979).

Exposure of mammalian ribosomes to media containing high concentrations of KCl has been proposed as a method for differentiating uncomplexed monoribosomes from ribosomes attached to messenger RNA (Martin & Hartwell, 1970). This procedure could not be employed in the present studies to distinguish between the phosphorylation states of 'vacant couples' and complexed monoribosomes in purified rat cerebral preparations. Thus exposure of these cerebral ribosomal preparations from either control or phenylalanine-treated rats to media containing 500 mM-KCl resulted in partial dissociation of polyribosomes as well as monoribosomes (S. Roberts & B. S. Morelos, unpublished work). This phenomenon was noted with preparations containing both polyribosomes and monoribosomes after purification by centrifugation through 2 M-sucrose for 4 or 20 h in the presence of 100 mM-KCl, as well as with separated cerebral polyribosomes and monoribosomes derived from these preparations. It is unlikely that the sensitivity of purified cerebral ribosomes from control rats to high concentrations of KCl was related to damage incurred by the ribosomes during isolation, since these ribosomes contained a high proportion of large polyribosomes. The relative sensitivity of purified cerebral polyribosomes from normal rats to alterations in the ionic composition of the suspending medium has been described previously, and evidence has been presented that this instability was not due to exposure of the ribosomes to ribonuclease action during isolation (Zomzely *et al.*, 1966, 1968; Zomzely-Neurath & Roberts, 1972). It must also be noted that, although certain mammalian polyribosomes may not be substantially altered simply by exposure to media containing high concentrations of KCl, polyribosomes from other mammalian cells are readily dissociated by this procedure (Traugh & Traut, 1973).

Variations in the state of aggregation of cerebral ribosomes may be responsible for the altered distribution of phosphorylated congeners of the S6 ribosomal protein between polyribosomes and monoribosomes in phenylalanine-treated infant rats. However, the diminished phosphorylation state of the S6 protein in separated cerebral polyribosomes under these circumstances could not be explained simply by this mechanism. One possible explanation

for the latter phenomenon is suggested by the finding that the decreased phosphorylation state of the S6 ribosomal protein that occurs in rat liver after parenteral administration of methionine (Treloar *et al.*, 1977) is preceded by a decrease in hepatic ATP concentrations (Farber, 1967). In the present studies, diminished labelling of the cerebral S6 protein might also be related to decreased availability of ATP. Thus, high concentrations of L-phenylalanine have been shown to inhibit pyruvate kinase activity in cell-free preparations of rat brain cortex, and it has been postulated that this inhibition could lead to decreased production of ATP (Weber, 1969). Since phosphorylation of the S6 protein accounts for 80–90% of the total phosphate turnover of cerebral ribosomal proteins *in vivo* (Roberts & Morelos, 1979), it is possible that decreased availability of cerebral ATP would result in selective inhibition of the labelling of this protein. Phenylalanine loading may also directly inhibit cerebral protein kinases responsible for phosphorylation of the S6 ribosomal protein, but no evidence is available to support this hypothesis. Another possible explanation for decreased phosphorylation of the cerebral S6 protein in phenylalanine-treated rats is suggested by the observation that the increase in acid ribonuclease activities in the cerebral cytosol under these circumstances appears to be associated with structural labilization of lysosomes (Roberts, 1977). It is possible that additional enzymes of lysosomal origin are involved in producing alterations in the structure and function of cerebral ribosomes *in vivo* in experimental hyperphenylalaninaemia. In this regard, phosphoprotein phosphatases of lysosomal origin have been described in mammalian tissues (Tappel, 1969; Barrett, 1969, 1972), but the capacity of these phosphatases to dephosphorylate ribosomal proteins has not yet been determined.

In view of the fact that the functional roles of ribosomal protein phosphorylation are unknown, the precise significance of the present observations cannot be determined at this time. Several lines of evidence suggest that alterations in phosphorylation of the S6 ribosomal protein may result in variations in polyribosome formation and stability, as well as translational activity. Thus, data from cross-linking experiments suggest that the S6 protein is involved in the attachment of messenger RNA to the 40S ribosomal subunit in eukaryotic cells (Anderson *et al.*, 1977; Terao & Ogata, 1979). Moreover, increased phosphorylation of the S6 protein has been shown to accompany increases in polyribosome formation and protein synthesis in mouse fibroblast cells in culture after stimulation with foetal calf serum (Thomas *et al.*, 1979). In contrast, recent experiments with mouse myeloma cells grown in a hyper-osmotic medium indicate that the resulting

block in initiation is associated with decreased phosphorylation of the S6 ribosomal protein (Martini & Kruppa, 1979). In the present investigations, it is possible that differences in the phosphorylation state of the cerebral S6 ribosomal protein could favour utilization of ribosomal subunits for protein synthesis in control animals, and contribute to an inhibition of polyribosome re-formation in hyperphenylalaninaemic animals.

Evidence has been presented that each ribosome possesses only one copy of each of the various congeners of the S6 protein (Gressner & Wool, 1976), and the proportions of these congeners in neural tissue vary considerably depending on the physiological state (Roberts & Ashby, 1978; Roberts & Morelos, 1979). These considerations suggest that specific alterations in phosphorylation of the S6 protein could selectively influence the ability of the ribosomal population to translate certain messenger RNA species in the brain. In experimental hyperamino-acidaemias and the human amino-acidopathies, synthesis of myelin proteins or other specific brain proteins may be selectively altered by this mechanism.

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