Inhibition of cerebral protein kinase activity and cyclic AMP-dependent ribosomal-protein phosphorylation in experimental hyperphenylalaninaemia

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Studies were carried out to elucidate the mechanisms underlying the diminished phosphorylation of cerebral ribosomal protein in experimental hyperphenylalaninaemia [Roberts & Morelos (1980) *Biochem. J.* **190**, 405–419]. Administration of N^6,O^2' -dibutyryl cyclic AMP or 3-isobutyl-1-methylxanthine, which increased phosphorylation of the S6 protein of cerebral 40S ribosomal subunits in control infant rats, did not counteract the decreased phosphorylation of this ribosomal protein resulting from intraperitoneal administration of a loading dose of L-phenylalanine. N^2,O^2' -Dibutyryl cyclic GMP had no effect on cerebral ribosomal-protein phosphorylation in either control or hyperphenylalaninaemic animals. The phenylalanine-induced decrease in ribosomal-protein phosphorylation was associated with decreased protein kinase activity in cerebral cytosolic and microsomal preparations. However, the maximal protein kinase response to cyclic AMP added *in vitro* was unaltered by prior administration of phenylalanine *in vivo*. The heat-stable protein inhibitor of cyclic AMP-dependent protein kinases decreased the activity of these enzymes by about 90% and eliminated the phenylalanine-induced difference in protein kinase activity in the absence of added cyclic AMP. Intracisternal administration of doses of dibutyryl cyclic AMP or 3-isobutyl-1-methylxanthine which increased the cyclic AMP-dependent protein kinase activity ratio in control infant rats was without effect on this index in phenylalanine-treated animals. Dibutyryl cyclic GMP had no effect on the protein kinase activity ratio in either group of animals. These results suggest that inhibition of cerebral cyclic AMP-dependent protein kinases by abnormally high concentrations of phenylalanine may contribute to the decrease in cerebral ribosomal-protein phosphorylation in experimental hyperphenylalaninaemia.

The observation that myelination is defective in the central nervous system in certain of the human aminoacidopathies has led to exploration of possible derangements in brain protein synthesis in experimental models of these diseases (Gaulle *et al.*, 1975). Although several observations *in vivo* and *in vitro* indicate that the synthesis of myelin and possibly other brain proteins is altered in experimental hyperphenylalaninaemia (Agrawal *et al.*, 1970; Berger *et al.*, 1980), the biochemical processes underlying these alterations have not been elucidated (Roberts, 1977; Hughes & Johnson, 1978a). Investigations in our laboratory revealed that phosphorylation of the S6 protein of the cerebral 40S ribosomal subunit is acutely decreased by phenylalanine loading in infant rats (Roberts & Morelos, 1980). Phosphorylation of this protein in eukaryotic cells is normally stimulated by cyclic AMP (Wool,

1979), and also appears to be closely coupled with the activation of protein synthesis (Martini & Kruppa, 1979; Wettenhall & Howlett, 1979; Thomas *et al.*, 1980). The present experiments were designed to investigate the mechanisms involved in the phenylalanine-induced decrease in cerebral ribosomal-protein phosphorylation. The results indicate that this inhibition is associated with decreased activation of cerebral cyclic AMP-dependent protein kinases.

Experimental

Treatment of animals

Infant male rats of an inbred Sprague–Dawley strain, 6–8 days old and weighing 18–20 g, were used in these investigations. The animals were kept warm under an incandescent lamp throughout the experiment.

Phosphorylated ribosomal proteins in cerebral cortices of these animals were labelled by intracisternal administration of 2mCi of [32 P]P_i in 20 μ l of 0.9% NaCl at pH7. L-Phenylalanine was administered intraperitoneally as a 3.0% (w/v) solution in 0.45% (w/v) NaCl (adjusted to pH7) in amounts equivalent to 2mg/g body wt. Dibutyl cyclic AMP, dibutyl cyclic GMP and 3-isobutyl-1-methylxanthine were administered intracisternally. Solutions of these substances were adjusted to pH7 before administration. Administration of equivalent volumes of 0.9% (w/v) NaCl to infant rats intracisternally or intraperitoneally had no discernible effect on patterns of cerebral ribosomal proteins obtained by two-dimensional gel electrophoresis (Roberts & Morelos, 1979), sedimentation of purified cerebral ribosomes (Roberts & Morelos, 1976), or cerebral protein kinase activities (see the Results section).

The animals were killed by decapitation or under light anaesthesia with pentobarbital sodium. The 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 dissected free of underlying tissue, weighed on a torsion balance, and homogenized as described below.

Preparation of cerebral ribosomes and ribosomal subunits

The methods employed to prepare purified cerebral ribosomes and ribosomal subunits for sucrose-density-gradient analysis and isolation of ribosomal proteins have been described in detail (Roberts & Morelos, 1980). Cerebral tissue was gently 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, pH7.6 at 4°C, and ribonuclease inhibitor protein (50 μ g/ml) (Roberts & Morelos, 1976). The homogenates were centrifuged at 8000 g for 10 min at 0°C to obtain the post-mitochondrial-supernatant fraction used to prepare cerebral ribosomes. Samples (0.1 ml) of the post-mitochondrial-supernatant preparation were treated with 0.9 ml of 10% (w/v) trichloroacetic acid. After centrifugation, radioactivity in the supernatant fraction was determined as a measure of 32 P uptake into the cerebral cytosol (Roberts & Morelos, 1980).

Two-dimensional electrophoresis of cerebral ribosomal proteins

Ribosomal proteins were isolated from purified 40S and 60S subunits and subjected to two-dimensional slab-gel electrophoresis as described previously (Roberts & Ashby, 1978; Roberts & Morelos, 1980). Samples were also taken for measurement of protein and radioactivity (Roberts & Morelos, 1980). The slabs were stained with

Coomassie Brilliant Blue. In some cases, the region of the wet gel corresponding to the S6 protein was excised, scanned at 600nm, and cut into 1mm sections for scintillation counting after solubilization in 30% H₂O₂ (Ashby & Roberts, 1975). In other instances, the gel slabs were dried, photographed, and subjected to radioautography against Kodak No-Screen X-ray film.

Measurement of cerebral protein kinase activity

Histone kinase activity was measured in crude cerebral extracts, as well as separated cytosolic and microsomal fractions, in the presence and absence of cyclic AMP. The procedures routinely used for tissue homogenization and enzyme assay were similar to those described by Palmer *et al.* (1980). The method of Hofmann *et al.* (1977) was also used in some experiments (see the Results section). In the routine procedure, cerebral cortices from four to six animals in each group were homogenized at 4°C in buffer (1:20; w/v) composed of 10 mM-potassium phosphate, pH6.8, 0.5 M-NaCl, 15 mM- β -mercaptoethanol, 10 mM-EDTA, 0.5 mM-3-isobutyl-1-methylxanthine, and Norite (10 mg/ml). Use of this homogenization medium minimizes dissociation of the cyclic nucleotide-dependent holoenzymes (Keely *et al.*, 1975), as well as reassociation of the type II enzyme (Corbin *et al.*, 1973), which predominates over the type I isoenzyme in rat brain (Corbin *et al.*, 1975; Francis & Roberts, 1978). The cerebral homogenate was centrifuged at 25 000 g and 0°C for 8 min to obtain a postmitochondrial-supernatant fraction (crude extract). In some experiments, a portion of this fraction was centrifuged at 226 400 g for 30 min at 0°C to prepare cytosolic and microsomal fractions. The microsomal fraction was resuspended in homogenization medium. Immediately after preparation, each fraction was diluted with homogenization medium and assayed for protein kinase activity. Samples were also taken in duplicate for measurement of protein.

The routine assay medium was composed of 27 mM-Pipes (1,4-piperazinediethanesulphonic acid) (sodium salt), pH6.8, 4 mM-MgCl₂, 0.27 mM-EGTA, 0.133 mM-[γ - 32 P]ATP (approx. 500 c.p.m./pmol), 0.6 mg of histone type II-A (when added), 5 or 10 μ M-cyclic AMP (when added), and 3–6 μ g of brain protein in a final volume of 75 μ l. Addition of the phosphoprotein phosphatase inhibitors NaF (10–40 mM) or sodium molybdate (20 mM) to the extraction and/or assay media did not increase cerebral protein kinase activities. Incubations were routinely performed in quadruplicate at 30°C for 5 min, but linear kinetics were obtained for incubation periods of 2–15 min with amounts of enzyme protein of 2–10 μ g (results not shown). After incubation, 60 μ l of assay mixture were quickly pipetted on to a Whatman 3 MM filter-paper disc

(23 mm diam.). The disc was immediately placed in a wire basket suspended in 500 ml of ice-cold 10% trichloroacetic acid and the solution was stirred with a magnetic stirrer for at least 10 min. The basket was then transferred to 500 ml of 10% trichloroacetic acid containing 1 ml of phosphoric acid/litre and the solution was heated at 90°C for 30 min to hydrolyse excess [γ - 32 P]ATP. The discs were next washed twice in 500 ml of 10% trichloroacetic acid containing phosphoric acid to facilitate removal of released [32 P]phosphate, then once in 500 ml of 95% (v/v) ethanol and once in 500 ml of diethyl ether, each for 10 min. Finally, the discs were dried in air, and placed in scintillation vials with 9 ml of 3a70B scintillation cocktail for determination of 32 P radioactivity incorporated into protein.

Protein analyses

Protein contents of the various cerebral-cortical preparations and ribosomal-protein samples were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

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. [32 P]P_i (285 Ci/mg; carrier-free), obtained from ICN Pharmaceuticals, Irvine, CA, U.S.A., was supplemented with NaCl to a final concentration of 0.9% (w/v) NaCl just before intracisternal administration. [γ - 32 P]ATP was prepared essentially as described by Glynn & Chappell (1964). 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 the protein inhibitor of cyclic AMP-dependent protein kinase from ox heart, puromycin hydrochloride, histone F2b, histone type II-A, β -mercaptoethanol, bovine serum albumin and the sodium salts of *N*⁶,*O*^{2'}-dibutyryl cyclic AMP and *N*²,*O*^{2'}-dibutyryl cyclic GMP. Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) supplied 3-isobutyl-1-methylxanthine. L-Phenylalanine (A grade), Mes (4-morpholine-ethanesulphonic acid) and Pipes (sodium salt) 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. Norite-A was purchased from Matheson, Coleman and Bell, Norwich, OH, U.S.A. Scintillation-counting solution (3a70B) was obtained from Research Products International, Elk Grove Village, IL, U.S.A.

Results

Influence of cyclic nucleotides on phenylalanine-induced inhibition of ribosomal-protein phosphorylation in rat cerebral cortex in vivo

Incorporation of radioactivity from intracisternally injected [32 P]P_i into cerebral 40S ribosomal protein of infant rats *in vivo* has been shown to be selectively stimulated by parenteral administration of dibutyryl cyclic AMP or the potent inhibitor of cyclic nucleotide phosphodiesterase, 3-isobutyl-1-methylxanthine (Roberts & Morelos, 1979). In contrast, intraperitoneal administration of loading doses of L-phenylalanine markedly decreased incorporation of 32 P into cerebral 40S ribosomal protein (Roberts & Morelos, 1980). The present investigations reveal that this inhibitory effect of phenylalanine loading on cerebral ribosomal-protein phosphorylation could not be overcome by intracisternal administration of doses of dibutyryl cyclic AMP or 3-isobutyl-1-methylxanthine that stimulated this phosphorylation process in control animals (Table 1). Dibutyryl cyclic GMP was without effect on the phosphorylation process in either control or phenylalanine-treated animals. Uptake of 32 P into the cerebral cytosol was not significantly altered under any of these circumstances.

Two-dimensional electrophoresis of ribosomal proteins isolated from the cerebral small ribosomal subunit of infant rats indicated that the variations in 32 P incorporation into cerebral 40S ribosomal protein in response to phenylalanine loading and the administration of dibutyryl cyclic AMP were associated with alterations in the proportions of the more highly phosphorylated congeners of the S6 protein, which travelled more slowly at pH 8.6 in the first dimension than did the non-phosphorylated form (Fig. 1). Thus the stimulatory effect of dibutyryl cyclic AMP was observed in both electrophoretograms stained for protein (cf. Figs. 1*b* and 1*a*) and radioautographs (cf. Figs. 1*f* and 1*e*) as increases in the relative proportions of stained and radioactive material in the more acidic components of the S6 protein. In contrast, intraperitoneal administration of a loading dose of L-phenylalanine decreased phosphorylation of the S6 protein, as judged by these same indices (Figs. 1*c* and 1*g*). Subsequent administration of dibutyryl cyclic AMP had little or no effect on phosphorylation of the cerebral S6 protein in phenylalanine-treated animals (Figs. 1*d* and 1*h*). Comparable results were obtained when 3-isobutyl-1-methylxanthine was substituted for dibutyryl cyclic AMP (Fig. 2). In control animals, the radioactivity in the S6-protein region was associated with four or five protein species on the gel (Fig. 2*a*). An additional congener of the S6 protein, which moved most rapidly in the first dimension at pH 8.6, was non-radioactive. Administration of

Table 1. *Influence of cyclic nucleotides and phenylalanine loading on phosphorylation of 40S ribosomal proteins in rat cerebral cortex in vivo*

Infant rats were injected intraperitoneally with L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. All animals were given 2 mCi of [32 P]P_i intracisternally in 20 μ l of 0.9% NaCl 30 min later. The cyclic nucleotides (25 μ g/rat) and 3-isobutyl-1-methylxanthine (10 μ g/rat) were administered intracisternally in conjunction with the radioactive isotope. The animals were killed 1 h after the intracisternal injection. Postmitochondrial-supernatant fractions from cerebral cortices of 20 rats in each group were pooled in sets of five to give four homologous samples for measurements of 32 P uptake. The remainders of these fractions from similarly treated animals were then combined. Free ribosomes were isolated and dissociated in the presence of high concentrations of KCl. Purified 40S ribosomal proteins were dissolved in 150 μ l of 8 M-urea containing 10 mM- β -mercaptoethanol for subsequent analysis by two-dimensional gel electrophoresis. Samples (10 μ l) were taken for determination of protein and radioactivity. Measured radioactivities were corrected for radioactive decay from the time of administration of radioisotope.

Treatment	$10^{-5} \times ^{32}\text{P}$ uptake (c.p.m./g of cortex)*	Specific radioactivity (c.p.m./ μ g of 40S-subunit proteins)
Expt. 1		
Control	3406 $\pm$ 302	132.0
Dibutyryl cyclic AMP	3396 $\pm$ 366	170.3
Phenylalanine	3363 $\pm$ 351	73.6
Phenylalanine + dibutyryl cyclic AMP	3187 $\pm$ 149	63.9
Expt. 2		
Control	2832 $\pm$ 291	104.5
3-Isobutyl-1-methylxanthine	2591 $\pm$ 174	149.5
Phenylalanine	2737 $\pm$ 239	53.6
Phenylalanine + 3-isobutyl-1-methylxanthine	2334 $\pm$ 210	49.0
Expt. 3		
Control	2918 $\pm$ 185	118.2
Dibutyryl cyclic GMP	3222 $\pm$ 332	99.1
Phenylalanine	2695 $\pm$ 264	65.5
Phenylalanine + dibutyryl cyclic GMP	2836 $\pm$ 345	57.7

* Average $\pm$ S.E.M. for four separate samples of pooled postmitochondrial-supernatant fractions from five cerebral cortices.

Fig. 1. *Influence of dibutyryl cyclic AMP and phenylalanine loading on two-dimensional electrophoretograms of proteins isolated from 40S ribosomal subunits of rat cerebral cortex*

Infant rats were injected intraperitoneally with L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. The animals were given 2 mCi of [32 P]P_i intracisternally in 20 μ l of 0.9% NaCl 30 min later. Dibutyryl cyclic AMP was administered intracisternally (25 μ g/rat) in conjunction with the radioactive isotope. All animals were killed 1 h after the injection of radioisotope. Proteins extracted from cerebral 40S ribosomal subunits from 20 rats in each group were subjected to two-dimensional electrophoresis on polyacrylamide gels containing urea. The amount of ribosomal protein applied to each of the first-dimensional gels was approx. 400 μ g. (a), (b), (c) and (d), Electrophoretograms stained with Coomassie Brilliant Blue; (e), (f), (g) and (h), radioautographs exposed for 1 day. (a) and (e), Control; (b) and (f), dibutyryl cyclic AMP; (c) and (g), phenylalanine; (d) and (h), phenylalanine + dibutyryl cyclic AMP.

3-isobutyl-1-methylxanthine increased the relative amounts of the phosphorylated congeners of the S6 protein, as judged by changes in both the absorbance profile and the radioactivity distribution (Fig. 2b). In contrast, phenylalanine loading inhibited phosphorylation of this protein (Fig. 2c), and subsequent administration of 3-isobutyl-1-methylxanthine did not overcome this inhibition (Fig. 2d).

Influence of phenylalanine loading on cyclic AMP-dependent protein kinase in rat cerebral cortex

The observations described above indicated that decreased phosphorylation of the S6 ribosomal protein in cerebral cortices of hyperphenylalaninaemic infant rats was associated with decreased stimulation of this process by exogenously administered or endogenously produced cyclic AMP. Since phosphorylation of the cerebral S6 protein is at least partly regulated by cyclic AMP-dependent protein kinases (Roberts & Ashby, 1978; Roberts & Morelos, 1979; Francis & Roberts, 1980), experiments were carried out to determine the effect of

phenylalanine loading on these enzymes. Intraperitoneal administration of loading doses of L-phenylalanine to infant rats resulted within 60 min in significant decreases in histone kinase activity in crude extracts of the rat cerebrum, when this activity was measured in the absence of added cyclic AMP (Table 2). This effect could frequently be detected within 30 min after injection of a loading dose of the amino acid, and was continued for about 2 h (results not shown). Histone kinase activity measured in the presence of cyclic AMP was not significantly altered by phenylalanine loading, with the result that the protein kinase activity ratio was decreased under these circumstances.

Administration of dibutyryl cyclic AMP intracisternally (25 μ g) increased the cyclic AMP-dependent protein kinase activity ratio in cerebral cortices of otherwise untreated infant rats (Table 2, Expts. 1 and 2). In contrast, dibutyryl cyclic AMP had little or no effect on this activity ratio in the cerebri of hyperphenylalaninaemic infant rats. Comparable results were obtained with animals killed either by

Fig. 2. Influence of 3-isobutyl-1-methylxanthine and phenylalanine loading on phosphorylation of the S6 ribosomal protein in rat cerebral cortex *in vivo*

Infant rats were injected intraperitoneally with L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. All animals were given 2 mCi of [32 P] P_i intracisternally in 20 μ l of 0.9% NaCl 30 min later. 3-Isobutyl-1-methylxanthine was administered intracisternally (10 μ g/rat) in conjunction with the radioactive isotope. The animals were killed 1 h after the injection of radioisotope. The amount of 40S ribosomal protein applied to each of the first dimensional gels was approx. 400 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 derivatives was cut out and scanned for A_{600} (—). This section of the gel was then divided into 1 mm slices for determination of radioactivity (●). Radioactivity measurements were not corrected for decay. (a) Control; (b) 3-isobutyl-1-methylxanthine; (c) phenylalanine; (d) phenylalanine + 3-isobutyl-1-methylxanthine.

Table 2. Influence of prior administration of cyclic nucleotides and phenylalanine loading on cyclic AMP-dependent histone kinase activity in crude extracts of rat cerebral cortex

Infants rats were injected intraperitoneally with L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. The dibutyryl derivative of cyclic AMP or cyclic GMP (25 µg/rat), 3-isobutyl-1-methylxanthine (10 µg/rat) or 0.9% NaCl was given intracisternally in 20 µl of 0.9% NaCl 30 min later. All animals were killed 30 min after this second injection, by decapitation in Expts. 1, 3 and 4, and under light anaesthesia with pentobarbital sodium (2 mg/100 g body wt., in 0.9% NaCl, given subcutaneously) in Expt. 2. Cerebral crude extracts were prepared and assayed for protein kinase activity as described in the text. Protein phosphorylation in the absence of histone was subtracted from total protein phosphorylation to provide a measure of histone kinase activity. The activity ratios are calculated as the ratio of histone kinase activity in the absence of cyclic AMP to activity in the presence of optimal concentrations of cyclic AMP (Corbin *et al.*, 1973). Histone kinase activity is expressed as pmol of P_i transferred from [γ -³²P]ATP to protein/min per mg of tissue protein; averages $\pm$ S.E.M. for four determinations are shown.

Treatment	Histone kinase activity (pmol of P _i /min per mg of protein)		Activity ratio
	–cyclic AMP	+cyclic AMP	
Expt. 1			
Control	248 $\pm$ 13	413 $\pm$ 16	0.60
Dibutyryl cyclic AMP	310 $\pm$ 13	420 $\pm$ 18	0.74
Phenylalanine	185 $\pm$ 6	410 $\pm$ 15	0.45
Phenylalanine + dibutyryl cyclic AMP	192 $\pm$ 11	416 $\pm$ 11	0.46
Expt. 2			
Control	230 $\pm$ 12	426 $\pm$ 10	0.54
Dibutyryl cyclic AMP	306 $\pm$ 14	438 $\pm$ 11	0.70
Phenylalanine	176 $\pm$ 10	445 $\pm$ 16	0.40
Phenylalanine + dibutyryl cyclic AMP	179 $\pm$ 8	471 $\pm$ 16	0.38
Expt. 3			
Control	270 $\pm$ 10	473 $\pm$ 12	0.57
3-Isobutyl-1-methylxanthine	335 $\pm$ 13	459 $\pm$ 14	0.73
Phenylalanine	194 $\pm$ 10	486 $\pm$ 15	0.40
Phenylalanine + 3-isobutyl-1-methylxanthine	201 $\pm$ 10	426 $\pm$ 11	0.47
Expt. 4			
Control	279 $\pm$ 12	507 $\pm$ 18	0.55
Dibutyryl cyclic GMP	267 $\pm$ 11	515 $\pm$ 21	0.52
Phenylalanine	206 $\pm$ 14	541 $\pm$ 13	0.38
Phenylalanine + dibutyryl cyclic GMP	201 $\pm$ 13	492 $\pm$ 12	0.41

decapitation (Expt. 1) or under light anaesthesia with pentobarbital sodium (Expt. 2), even though the latter procedure prevents the increase in cyclic nucleotide concentrations in the brain that results from decapitation (Daly, 1977). In common with dibutyryl cyclic AMP, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, given intracisternally (10 µg/rat), increased the cyclic AMP-dependent protein kinase activity ratio in cerebral cortices of control animals, but was without effect on this index in phenylalanine-treated rats (Expt. 3). Intracisternal administration of dibutyryl cyclic GMP had no discernible effect on cerebral histone kinase activity in either control or hyperphenylalaninaemic animals, even at twice the dose shown (Expt. 4).

Protein kinase activities in cerebral cytosolic and microsomal fractions in the absence of added cyclic AMP were also increased by parenteral administration of dibutyryl cyclic AMP and decreased by

phenylalanine treatment (Table 3). Moreover, in common with the results obtained with crude cerebral extracts, prior treatment of infant rats with phenylalanine prevented the stimulatory action of the cyclic nucleotide on protein kinase activity in cytosolic and microsomal fractions. Protein kinase activity in these cerebral subcellular fractions in the presence of added cyclic AMP was not altered by prior treatment of the animals with phenylalanine or dibutyryl cyclic AMP. These results are consistent with the conclusion that cytosolic and membrane-associated cyclic AMP-dependent protein kinases in cerebral tissue are very similar or identical (Rubin *et al.*, 1979).

Investigations of cerebral protein kinase activity were also carried out in the presence of the specific protein inhibitor of the cyclic AMP-dependent enzymes (Ashby & Walsh, 1972). When cerebral cell-free preparations from control and phenyl-

Table 3. Influence of prior administration of cyclic AMP and phenylalanine loading on cyclic AMP-dependent protein kinase activity in cytosolic and microsomal fractions of rat cerebral cortex

Infant rats were injected intraperitoneally with L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. The cyclic nucleotide (25 µg/rat) or 0.9% NaCl was given intracisternally in 20 µl of 0.9% NaCl 30 min later. All animals were killed 30 min after this second injection. Cerebral cytosolic and microsomal fractions were prepared as described in the text and immediately assayed for histone kinase activity. See legend to Table 2 for additional explanations.

Treatment	Cytosolic fraction			Microsomal fraction		
	Histone kinase activity (pmol of P _i /min per mg of protein)		Activity ratio	Histone kinase activity (pmol of P _i /min per mg of protein)		Activity ratio
	-cyclic AMP	+cyclic AMP		-cyclic AMP	+cyclic AMP	
Control	233 ± 14	479 ± 11	0.49	165 ± 12	391 ± 18	0.42
Dibutyryl cyclic AMP	307 ± 12	482 ± 10	0.64	218 ± 9	389 ± 14	0.56
Phenylalanine	158 ± 10	443 ± 19	0.36	94 ± 14	377 ± 16	0.25
Phenylalanine + dibutyryl cyclic AMP	160 ± 13	489 ± 17	0.33	108 ± 15	386 ± 13	0.28

Fig. 3. Influence of the protein inhibitor of cyclic AMP-dependent protein kinase on histone kinase activity in crude extracts of cerebral cortex from control and hyperphenylalaninaemic infant rats

Infant rats were injected intraperitoneally with L-phenylalanine (2 mg/g body wt.) in 0.45% NaCl, or an equivalent volume of 0.9% NaCl. The animals were killed 1 h later. Cerebral crude extracts were prepared and samples containing approx. 2 µg of protein were immediately assayed for histone kinase activity essentially as described by Hofmann *et al.* (1977). The assay medium also contained various amounts of protein kinase inhibitor. Incubation was for 2 min at 30°C. Symbols: cerebral extracts from saline-treated rats assayed in the presence (●) or the absence (○) of 1 µM-cyclic AMP; cerebral extracts from phenylalanine-treated rats assayed in the presence (■) or the absence (□) of 1 µM-cyclic AMP.

alanine-treated animals were assayed under conditions which resulted in very low phosphorylation of endogenous protein (Hofmann *et al.*, 1977), about 90% of the histone kinase activity measured in the presence of cyclic AMP could be eliminated by the inhibitor (Fig. 3). The protein inhibitor of cyclic AMP-dependent protein kinases also decreased the basal protein kinase activity in cerebral extracts from control and hyperphenylalaninaemic animals to the same low value observed in the presence of added cyclic AMP. These results imply that phenylalanine inhibition of cerebral protein kinase activity *in vivo* is due mainly to an action on cyclic AMP-dependent protein kinases.

Discussion

The results of numerous observations indicate that multiple aberrations in brain metabolism contribute to the neuropathological consequences of the hyperaminoacidaemias. Nevertheless, a common feature of several of these diseases involves defects in the metabolism of myelin proteins and other brain proteins (Gaull *et al.*, 1975). Although the mechanisms that underlie this phenomenon have not been elucidated, available evidence suggests that alterations in translational processes in brain protein synthesis may be involved. Thus, variations in ribosome structure (Roberts, 1977), as well as soluble components of the initiation process (Hughes & Johnson, 1978b), have been suggested as possible causes of the derangements in brain protein-synthesizing systems observed in experimental hyperphenylalaninaemia.

Earlier investigations from our laboratory revealed that phenylalanine loading in infant rats resulted in marked inhibition of phosphorylation of the S6 protein of the 40S ribosomal subunit in

cerebral polyribosomes (Roberts & Morelos, 1980). Phosphorylation of multiple sites in this protein normally accounts for approx. 90% of the total radioactivity incorporated into serine and threonine residues of constitutive ribosomal proteins of rat cerebral cortex *in vivo* after intracisternal administration of [32 P]P_i (Roberts & Morelos, 1979). Phosphorylation of the S6 ribosomal protein in eukaryotic cells has been shown to be modulated by cyclic AMP-dependent protein kinases (Gressner & Wool, 1976; Grankowski *et al.*, 1979; Roberts & Morelos, 1979; Francis & Roberts, 1980). However, other protein kinases may participate in this regulation (Del Grande & Traugh, 1979; Mumby & Traugh, 1979; Issinger *et al.*, 1980). Moreover, processes which are associated with alterations in protein synthesis, and which do not seem to involve cyclic AMP, are also capable of modifying phosphorylation of the S6 protein (Leader & Coia, 1978; Wettenhall & Howlett, 1979; Lastick & McConkey, 1980; Thomas *et al.*, 1980). Different sites within the S6 molecule appear to be phosphorylated in response to cyclic AMP and other agents which are capable of activating protein synthesis (Lastick & McConkey, 1981).

The present experiments indicate that decreased phosphorylation of the S6 ribosomal protein in cerebral cortices of hyperphenylalaninaemic infant rats is associated with insensitivity of this phosphorylation process to administered dibutyryl cyclic AMP or 3-isobutyl-1-methylxanthine. These compounds are normally capable of stimulating S6-protein phosphorylation in rat cerebral cortex *in vivo* and *in vitro*, whereas dibutyryl cyclic GMP is inactive in this regard (Roberts & Ashby, 1978; Roberts & Morelos, 1979). Dibutyryl cyclic AMP and 3-isobutylxanthine also selectively activated protein kinase in the cerebrum of control rats, but were incapable of producing this effect in hyperphenylalaninaemic rats. Moreover, protein kinase activity in cerebral extracts in the absence of added cyclic AMP was significantly decreased by phenylalanine loading. The implication from these results, that phenylalanine loading interferes with the activation by cyclic AMP of the holoenzymes of cyclic AMP-dependent protein kinases in the brain, is supported by the finding that the specific protein inhibitor of the catalytic subunit of these enzymes decreased basal and cyclic AMP-stimulated histone kinase activities to the same low value in cerebral extracts from both control and hyperphenylalaninaemic animals.

The present data are consistent with the concept that the decreased ability of cyclic AMP to activate cerebral protein kinases *in vivo* in phenylalanine-treated infant animals is at least partly responsible for decreased phosphorylation of the S6 ribosomal protein under basal conditions, as well as

in the presence of elevated concentrations of cyclic AMP resulting from the administration of dibutyryl cyclic AMP or 3-isobutyl-1-methylxanthine. However, it is also possible that high concentrations of phenylalanine may decrease the availability of cerebral ATP (Weber, 1969) and that, by analogy with the glycogen synthase system (Mitchell *et al.*, 1980), limiting concentrations of ATP may decrease the availability of certain sites on the cerebral S6 protein to phosphorylation by cyclic AMP-dependent protein kinase. Phenylalanine-induced alterations in the activities of brain phosphoprotein phosphatases could also contribute to the diminished state of phosphorylation of the cerebral S6 ribosomal protein in hyperphenylalaninaemic infant rats (Roberts & Morelos, 1980).

Cyclic AMP, by virtue of its capacity to alter the structure and function of numerous phosphoproteins, is involved in the regulation of various phases of metabolic activity (Rubin & Rosen, 1975; Nimmo & Cohen, 1977; Walsh & Cooper, 1979; Weller, 1979). Accordingly, variations in the activities of brain cyclic AMP-dependent protein kinases in the presence of elevated concentrations of phenylalanine could result in several of the defects in brain function observed in experimental and human phenylketonuria. The contribution of alterations in phosphorylation of the cerebral S6 ribosomal protein to these processes remains to be elucidated. However, mounting evidence favours the hypothesis that the state of phosphorylation of the S6 protein may influence events in the initiation process of protein synthesis (Kramer *et al.*, 1977; Martini & Kruppa, 1979; Wettenhall & Howlett, 1979; Thomas *et al.*, 1980). This concept is supported by findings which suggest that the S6 protein is involved in the binding of mRNA and other components of the initiation complex to the 40S ribosomal subunit (Terao & Ogata, 1979; Bommer *et al.*, 1980). The present investigations are consistent with the possibility that phenylalanine-induced alterations in cyclic AMP-dependent phosphorylation of the S6 ribosomal protein in the brain may alter the synthesis of myelin proteins and other brain proteins and thus contribute to the neurological manifestations in hyperphenylalaninaemia.

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