Effects of *p*-Chlorophenylalanine and α -Methylphenylalanine on Amino Acid Uptake and Protein Synthesis in Mouse Neuroblastoma Cells

By CHESTER J. KELLY* and TERRY C. JOHNSON

Division of Biology, Kansas State University, Manhattan, KA 66506, U.S.A.

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The phenylalanine analogues *p*-chlorophenylalanine and α -methylphenylalanine were used to inhibit phenylalanine hydroxylase in animal models for phenylketonuria. The present report examines the effects of these analogues on the metabolism of neuroblastoma cells. *p*-Chlorophenylalanine inhibited growth and was toxic to neuroblastoma cells. Although *in vivo* this analogue increased cell monoribosomes by 42%, it did not significantly affect poly(U)-directed protein synthesis *in vitro*. *p*-Chlorophenylalanine did not compete with phenylalanine or tyrosine for aminoacylation of tRNA and was therefore not substituted for those amino acids in nascent polypeptides. The initial cellular uptake of various large neutral amino acids was inhibited by this analogue but did not affect the flux of amino acids already in the cell; this suggested that an alteration of the cell's amino acid pools was not responsible for the cytotoxicity of the analogues. In contrast with *p*-chlorophenylalanine, α -methylphenylalanine did not exert these direct toxic effects because the administration of α -methylphenylalanine *in vivo* did not affect brain polyribosomes and a comparable concentration of this analogue was neither growth inhibitory nor cytotoxic to neuroblastoma cells in culture. The suitability of each analogue as an inhibitor of phenylalanine hydroxylase in animal models for phenylketonuria is discussed.

Several experimental approaches have been used to create a suitable animal model for phenylketonuria. These approaches have included the induction of a hyperphenylalaninaemic state either by raising the phenylalanine in the diet (Boggs & Waisman, 1964), or by injecting phenylalanine by the intraperitoneal route (Aoki & Siegel, 1970; MacInnes & Schlesinger, 1971; Taub & Johnson, 1975). The introduction of phenylalanine causes a rapid 'disaggregation' of brain polyribosomes (Aoki & Siegel, 1970), probably through an inhibition of the initiation of protein synthesis at the ribosomal level (Taub & Johnson, 1975). High concentrations of phenylalanine alter brain cell amino acid concentrations *in vivo* (Roberts, 1967; Siegel *et al.*, 1971) and *in vitro* (Hughes & Johnson, 1976). In addition to the effects of phenylalanine on the uptake and efflux of amino acids (Cohen & Lajtha, 1972; Hughes & Johnson, 1976), high concentrations of the amino acid have been shown to be inhibitory to protein synthesis in brain cells *in vitro* (Roscoe *et al.*, 1968; Hughes & Johnson, 1976) and *in vivo* (Roberts, 1967; Agrawal *et al.*, 1970; Hughes & Johnson, 1977a,b).

Neither the increased dietary intake of phenylalanine nor the injection of the amino acid causes an inhibition of phenylalanine hydroxylase and, there-

fore, plasma tyrosine concentrations may rise abnormally. In order to overcome this limitation, and to create a model system more similar to the naturally occurring disease, the phenylalanine analogue *p*-chlorophenylalanine has been widely used to block hepatic phenylalanine hydroxylase activity (Lipton *et al.*, 1967; Watt & Martin, 1969; Guroff, 1969; Copenhaver *et al.*, 1973; Antonas & Coulson, 1975). Although this analogue effectively inhibits phenylalanine hydroxylase and maintains a high ratio of [phenylalanine]/[tyrosine] in the plasma, the action of the analogue is not specific to phenylalanine hydroxylase (Koe & Weissman, 1966). Therefore animals treated with *p*-chlorophenylalanine may show effects primarily associated with other metabolic events not solely related to the induced hyperphenylalaninaemia. Although these former studies have been useful to our understanding of the relationship between amino acid imbalances and neural function, results obtained from a non-specific model system should be interpreted with caution. This has been emphasized by the observation that *p*-chlorophenylalanine interferes with the growth of developing rats (Prohaska & Wells, 1974).

Another analogue of phenylalanine, α -methylphenylalanine, has been reported to be a potent inhibitor of hepatic phenylalanine hydroxylase and this compound appeared to be specific in its action (Greengard *et al.*, 1976). In addition, mice treated

* Present address: Department of Psychiatry, Michael Reese Hospital and Medical Center, Chicago, IL 60616, U.S.A.

with α -methylphenylalanine exhibit plasma phenylalanine concentrations approximately ten times higher than normal, without experiencing increased tyrosine concentrations or effects on the growth of the animals (Greengard *et al.*, 1976). Currently little information is available about the effects of either *p*-chlorophenylalanine or α -methylphenylalanine at the cellular level. We shall describe several features of these analogues on neuroblastoma cells at both the morphological and molecular level. In addition, the effects of these compounds on brain cell polyribosomes *in vivo* was examined as one measure of toxicity and as a method to discriminate between toxic effects of the analogues and those events actually associated with hyperphenylalaninaemia.

Materials and Methods

Materials

SCH:ARS (ICR)₁ non-inbred Swiss albino mice were purchased from ARS/Sprague-Dawley, Madison, WI, U.S.A. Ribonuclease-free sucrose was purchased from Schwarz/Mann, Orangeburg, NY, U.S.A. α -Methylphenylalanine was purchased from ICN Pharmaceuticals (Cleveland, OH, U.S.A.). All radioactive chemicals were obtained from New England Nuclear (Boston, MA, U.S.A.) Cell culture medium components were purchased from Grand Island Biological Co. (Grand Island, NY, U.S.A.) and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Incubation of neuroblastoma cells

Neuroblastoma cells (N2a), the kind gift of Dr. A. Telser of Northwestern University Medical School, were grown in monolayer culture in humidified incubators at 37°C, under a CO₂/air (1:19) atmosphere as described by Mathews *et al.* (1976). The cells were grown as a routine in 250 ml plastic culture flasks with 25 ml of Dulbecco's Modified Eagle's Medium (DME medium), supplemented with glucose (3.5 g/l), NaHCO₃ (3.7 g/l), antibiotics (penicillin, 100 units/ml; streptomycin, 100 µg/ml; fungizone, 0.25 µg/ml) and 10% γ -globulin-free newborn calf serum (Mathews *et al.*, 1976). Where indicated in the text, *p*-chlorophenylalanine or α -methylphenylalanine was added directly to the medium to a concentration of 5 mM. Uptake and efflux of radioactive amino acids were measured as described by Hughes & Johnson (1976).

Determination of total cellular protein

Plastic culture dishes (35 mm diameter) were inoculated with 1.5×10^5 cells with 2 ml of DME medium and were left to grow overnight at 37°C, after which the medium was replaced with medium made 5 mM with either *p*-chlorophenylalanine or

α -methylphenylalanine. At various intervals up to 48 h, the cells were scraped off the dish with a rubber 'policeman' and divided into two samples. One sample was used to measure both the number of cells in the culture with a haemocytometer and cell viability by Trypan Blue exclusion. Cellular protein was measured in the second sample, after the cells were pelleted by low-speed centrifugation (500g for 5 min), washed twice with 0.9% NaCl, and the final pellet was resuspended in 1 ml of 0.9% NaCl. The acid-insoluble material was precipitated with hot 5% (w/v) trichloroacetic acid, and the protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin used as the protein standard.

Sucrose-gradient-centrifugation analysis of polyribosomes

Neuroblastoma cells. Neuroblastoma cells were incubated at 37°C in regular DME medium or in DME medium containing either 5 mM *p*-chlorophenylalanine or 5 mM α -methylphenylalanine, and then cycloheximide was added to a final concentration of 0.5 mg/ml for 5 min to prevent run-off of the polyribosomes during isolation. The media was poured off, the cell monolayers were washed with ice-cold 0.9% NaCl and the cells were lysed with TKM buffer (50 mM-Tris/HCl, pH 7.2/25 mM-KCl/5 mM-MgCl₂) containing 0.25% (v/v) NP40 and bentonite (1 mg/ml). The lysates were centrifuged at 2000g to remove nuclei and the supernatant fluid was layered on 5.0 ml linear (0.7–1.5 M) sucrose/TKM buffer (w/v) gradients. They were then centrifuged at 190000g for 105 min in a Beckman L5-50 ultracentrifuge, model SW50.1 rotor, at 4°C as described by Taub & Johnson (1975). After centrifugation, the gradients were collected from the top with a peristaltic pump through a Buchler Densiflow apparatus and the A_{254} was continuously monitored with an ISCO model UA2 u.v. analyser.

Mouse brain. Mice were injected intraperitoneally with either *p*-chlorophenylalanine or α -methylphenylalanine at a concentration of 2 mg/g body wt. or with an equal volume of 0.9% NaCl. After 30–40 min, the brains were removed and homogenized in TKM buffer containing 1 mg of bentonite/ml (Lerner & Johnson, 1970). The homogenate was centrifuged at 8400g for 15 min at 4°C to remove mitochondria and cellular debris. The post-mitochondrial supernatant containing the polyribosomes was removed and 10% (w/v) sodium deoxycholate and Tween 40 were each added to a final concentration of 1%. The polyribosomes were purified by elution with TKM buffer through a column (1.1 cm $\times$ 10 cm) of Sephadex G-200 at 4°C and the fractions containing polyribosomes were pooled and analysed by sucrose gradient centrifugation (Taub & Johnson, 1975).

Protein synthesis in vitro

The pH 5 enzyme was isolated and protein synthesis was measured essentially as described by Lerner & Johnson (1970). The amount of radioactivity in the acid-insoluble material was determined in a Beckman LS-100 liquid-scintillation system as described by Johnson (1968). The amount of [^3H]phenylalanine incorporated into acid-insoluble proteins was corrected for control samples that were incubated at 0°C for 30 min and processed as described above.

Isolation of mouse brain tRNA and aminoacyl-tRNA synthetases

tRNA was isolated from young mice essentially as described by Hughes & Johnson (1977b).

Aminoacyl-tRNA synthetases were prepared from the post-microsomal fractions of mouse brain tissue as described by Johnson (1969) and modified by Johnson & Chou (1973).

Aminoacylation of tRNA

The aminoacylation of tRNA was carried out by a modification of the procedure described by Hughes & Johnson (1977b). Each reaction mixture (1.0 ml) contained: 20 μmol of Tris/HCl, pH 7.2; 14.3 μmol of 2-mercaptoethanol; 0.35–1.3 A_{260} units of tRNA; 0.18–0.32 mg of synthetase protein; 1–2 μCi of ^{14}C -labelled amino acid (phenylalanine or tyrosine); 1.5 mM-ATP and 0.2 mM-CTP. The reactions were incubated at 37°C for 40 min and the tRNA was precipitated with 2 vol. of ethanol after 0.1 ml each of glycogen (20 mg/ml) and 5 M-NaCl was added. The insoluble material was sedimented by low-speed centrifugation, repeatedly washed with ethanol, and collected on Millipore filters (0.45 μm). The amount of radioactivity was determined in a Beckman LS-100 liquid-scintillation system as described by Johnson (1968).

Results

In order to study the effects of the phenylalanine analogues *p*-chlorophenylalanine and α -methylphenylalanine at a cellular level with cells derived from the mammalian nervous system, we used the neuroblastoma N2a cell line. Neuroblastoma cells grown as monolayer cultures for 48 h typically extended numerous neurites (Fig. 1a). The effects of various concentrations of *p*-chlorophenylalanine, ranging from 2.5 to 10 mM, on cellular morphology and the ability of neuroblastoma N2a cells to extend neurites when freshly subcultured in the presence of this analogue were measured. Concentrations of *p*-chlorophenylalanine of 5 mM or higher markedly inhibited neurite extension, and within 48 h the N2a cells were generally spherical in shape and granulated

(Fig. 1b). Concentrations of *p*-chlorophenylalanine below 5 mM also exerted an alteration in cellular morphology, although the changes were less extensive and required longer periods of incubation to elicit this response. The effects of 5 mM-*p*-chlorophenylalanine on cell morphology were not restricted to active outgrowth of neurite extensions since the addition of this concentration of *p*-chlorophenylalanine to cells established as monolayer cultures (already with neurites) caused a retraction of the neurites, resulting in cell populations similar to those shown in Fig. 1(b). In addition to the significant change in cellular morphology, cultures incubated with *p*-chlorophenylalanine also appeared to have significantly fewer cells. In contrast with *p*-chlorophenylalanine, the presence of 5 mM- α -methylphenylalanine did not apparently affect neuroblastoma-cell morphology or the density of the cell population.

As *p*-chlorophenylalanine-treated cell cultures apparently had fewer cells during the incubation period, the effects of both *p*-chlorophenylalanine and α -methylphenylalanine on cell growth were measured by quantifying the total cellular protein and cell number per culture. Although control cultures of N2a cells grew exponentially, the presence of 5 mM-*p*-chlorophenylalanine clearly inhibited cell growth during a 48 h incubation period (Fig. 2). In contrast with *p*-chlorophenylalanine, cells grown in media containing 5 mM- α -methylphenylalanine were not inhibited in growth, and the increase in total cell protein per culture paralleled that of the controls. Similar results were obtained whether quantification was based on total cell protein or total cell count per culture. In addition to the effects on cell growth, cell viability was examined after cells had been incubated with either *p*-chlorophenylalanine or α -methylphenylalanine for 48 h. The presence of *p*-chlorophenylalanine resulted in only 24% cell viability as measured by Trypan Blue exclusion, although more than 95% of the cells were viable in the control cultures incubated in DME medium or DME medium containing α -methylphenylalanine. Therefore *p*-chlorophenylalanine both inhibited growth and was cytotoxic, but α -methylphenylalanine at this concentration apparently exerted neither of these effects.

Since the lack of an increase in cellular protein during incubation of the neuroblastoma cells with 5 mM-*p*-chlorophenylalanine may have resulted from an inhibition of cellular protein synthesis, the effects of both α -methylphenylalanine and *p*-chlorophenylalanine on cell polyribosome profiles were therefore measured. The amount of monoribosomes did not increase in cells incubated with 5 mM- α -methylphenylalanine for 90 min (Fig. 3b), although addition of 5 mM-*p*-chlorophenylalanine caused a rapid and large increase (Fig. 3c) when compared with the

(a)

(b)

Fig. 1. *Neuroblastoma cells incubated with p-chlorophenylalanine*
Plastic culture dishes (35 mm diameter) were inoculated with 1.5×10^5 cells/dish and the cells were grown for 48 h in either Dulbecco's Modified Eagle's Medium (DME) medium (a) or DME medium containing 5 mM *p*-chlorophenylalanine (b).

Fig. 2. Growth of neuroblastoma cells in the presence of either *p*-chlorophenylalanine or α -methylphenylalanine. Neuroblastoma cells were grown in DME medium (●), or DME medium containing 5mM-*p*-chlorophenylalanine (○) or 5mM- α -methylphenylalanine (Δ). At 4, 12, 24 and 48 h cells were scraped into ice-cold 0.9% NaCl. The total amount of protein per culture was determined as described in the Materials and Methods section.

Fig. 3. Sucrose-density-gradient analysis of neuroblastoma cell polyribosomes. Neuroblastoma cells were incubated in DME medium, or in DME medium containing 5mM-*p*-chlorophenylalanine or 5mM- α -methylphenylalanine. After 90 min the medium was removed and polyribosomes were analysed on a 1.5–0.7M-sucrose/TKM buffer gradient as described in the Materials and Methods section. (a) Polyribosomes isolated from cells incubated in DME medium. (b) Polyribosomes isolated from cells incubated in media containing 5mM- α -methyl-phenylalanine. (c) Polyribosomes isolated from cells incubated in DME medium containing 5mM-*p*-chlorophenylalanine. Sedimentation was from right to left and the arrows indicate the region of monoribosomes.

relative amount of monoribosomes isolated from control cultures incubated with DME medium (Fig. 3a). In further experiments, we found that the monoribosomes were dissociable into subunits by 500mM-KCl, which indicated that the monoribosomes were not active in protein synthesis and that they existed without mRNA or peptidyl-tRNA (Taub & Johnson, 1975). On a quantitative basis, monoribosomes increased by 42% in cells incubated in 5mM-*p*-chlorophenylalanine for only 30min. Cellular polyribosomes remained disrupted at this level for at least 90min.

To determine whether this increase in monoribosomes in cells incubated in 5mM-*p*-chlorophenylalanine did in fact represent an inhibition of protein synthesis, we measured the incorporation of radioactive amino acids into acid-insoluble material in the presence of *p*-chlorophenylalanine. Although there was a small decrease in the incorporation of radioactive amino acids into proteins, the results were difficult to interpret and quantify because the amounts of radioactive precursor in the intracellular acid-soluble pools were also decreased (results not shown). Therefore cell-free poly(U)-directed protein synthesis was carried out in the presence and in the absence of *p*-chlorophenylalanine to determine whether the analogue could directly disrupt polyphenylalanine synthesis at the ribosomal level. Although radioactive phenylalanine incorporation was decreased by 15% in the presence of the analogue (Table 1), the amount of inhibition was similar when *p*-chlorophenylalanine and radioactive phenylalanine were

Table 1. Effect of *p*-chlorophenylalanine on the incorporation of [³H]phenylalanine into polyphenylalanine. Poly(U)-directed protein synthesis was carried out in the presence of *p*-chlorophenylalanine. The concentration of [³H]phenylalanine was 0.75 pmol/reaction. Results are expressed as the average of duplicate determinations.

Expt. I	Concn. of <i>p</i> -chlorophenylalanine (pmol)	Acid-insoluble radioactivity	
		(c.p.m.)	% of control
Expt. I	None (control)	3065	100.0
	0.75	2440	79.6
	75.0	2740	89.4
Expt. II	None (control)	5420	100.0
	0.75	4890	90.2
	75.0	4290	79.2

present in equimolar concentrations and when *p*-chlorophenylalanine was added to a 100-fold excess. Therefore *p*-chlorophenylalanine was not competing with phenylalanine for incorporation into nascent polypeptides. To be certain that *p*-chlorophenylalanine was not being incorporated into nascent polypeptides, tRNA was aminoacylated *in vitro* with either [¹⁴C]phenylalanine or [¹⁴C]tyrosine,

the two amino acids most structurally similar to *p*-chlorophenylalanine. The amount of aminoacylation of the tRNA molecules was not affected by the presence of *p*-chlorophenylalanine at any of the concentrations of the analogue used (Table 2). The cytotoxicity of the analogue apparently was not associated with its acylation to these tRNA species and subsequent incorporation into neuroblastoma cell proteins.

Although the effects of *p*-chlorophenylalanine on ribosomal-directed protein synthesis were relatively small, *p*-chlorophenylalanine could have disrupted protein metabolism by affecting cellular uptake and efflux of amino acid precursors. For instance, high concentrations of phenylalanine itself, when incubated with brain cell suspensions, will disrupt the uptake kinetics of other large neutral amino acids (Hughes & Johnson, 1976). In order to determine whether *p*-chlorophenylalanine and α -methylphenylalanine could similarly affect neuroblastoma cells, the cells were incubated in DME medium or in DME medium containing either analogue at 5 mM. The cells were then incubated with either [3 H]phenylalanine, [3 H]tyrosine or [3 H]leucine, and the effects of the analogues on the initial rate of uptake of the amino acids was measured. The presence of 5 mM-*p*-chlorophenylalanine caused a decrease in uptake of all three amino acids (Table 3), but α -methylphenylalanine appeared to affect the uptake of each of the three amino acids quite differently. α -Methylphenylalanine apparently increased the initial rate of phenylalanine uptake, uptake of leucine was decreased and tyrosine transport remained essentially unaltered (Table 3). Neither of the compounds affected amino acid efflux when added to neuroblastoma cultures that were preincubated with either [3 H]-phenylalanine or [3 H]leucine (Table 4).

As both of these compounds have been used *in vivo* to produce a hyperphenylalaninaemic state, it was reasoned that results *in vivo* correlating with the

neuroblastoma-cell-culture studies may prove valuable. Animals were injected intraperitoneally with either 0.9% NaCl (controls) or 2 mg of each of the analogues per g body weight and the effects on neural polyribosomes were measured. Like phenylalanine itself (Taub & Johnson, 1975), *p*-chlorophenylalanine markedly increased brain monoribosomes (Table 5). Consistent with the cellular studies with α -methylphenylalanine, however, a similar dose of the analogue showed no direct toxic effects with regard to neural protein synthesis (Table 5).

Discussion

The cytotoxic effects of *p*-chlorophenylalanine on neuroblastoma cells were both rapid and persistent, since total cell protein decreased within 4 h of incubation and persisted for at least 48 h. Although the concentration of *p*-chlorophenylalanine in the media was approximately 10 times the concentration of other amino acids, e.g. phenylalanine, tyrosine and leucine, lower concentrations of the analogue may

Table 2. *Effect of p-chlorophenylalanine on the aminoacylation of tRNA in vitro with phenylalanine or tyrosine*

Mouse brain tRNA was aminoacylated in the presence or in the absence of *p*-chlorophenylalanine with either [14 C]phenylalanine or [14 C]tyrosine. The concentration of the radioactive amino acid in each case was 0.4 nmol/reaction. The results are expressed as the average of three independent determinations each done in triplicate $\pm$ S.E.M.

Concn. of <i>p</i> -chlorophenylalanine (nmol)	Phenylalanine (c.p.m.)	Tyrosine (c.p.m.)
None (control)	1210 $\pm$ 55	670 $\pm$ 38
0.4	—	770 $\pm$ 38
4.0	1610 $\pm$ 112	670 $\pm$ 42
40.0	1220 $\pm$ 42	760 $\pm$ 33

Table 3. *Effect of p-chlorophenylalanine and α -methylphenylalanine on the initial uptake of large neutral amino acids by neuroblastoma cells*

Neuroblastoma cells were incubated in media containing one of the 3 H-labelled amino acids indicated at a concentration of 2 μ Ci/ml. The culture media contained either *p*-chlorophenylalanine or α -methylphenylalanine at a concentration of 5 mM. The radioactivity represents the total cellular radioactivity and is expressed as the average of two independent determinations each done in duplicate.

Time of incubation (min)	Radioactivity (c.p.m./mg of cellular protein)								
	Phenylalanine			Tyrosine			Leucine		
	Control	<i>p</i> -Chloro- phenyl- alanine	α -Methyl- phenyl- alanine	Control	<i>p</i> -Chloro- phenyl- alanine	α -Methyl- phenyl- alanine	Control	<i>p</i> -Chloro- phenyl- alanine	α -Methyl- phenyl- alanine
1	13230	13320	21020	50100	30320	46400	30320	7070	25225
2	20140	12300	33480	58050	38520	54075	55140	19510	35635
3	19460	15140	31480	70060	45990	63070	56510	64160	37570

Table 4. *Effect of p-chlorophenylalanine and α-methylphenylalanine on the efflux of phenylalanine and leucine from neuroblastoma cells*

Neuroblastoma cells were incubated in media containing 2 μCi of either [³H]phenylalanine or [³H]leucine/ml. Either *p*-chlorophenylalanine or α-methylphenylalanine was then added to a final concentration of 5 mM (zero time). The radioactivity represents the total cellular radioactivity and is the average of two independent determinations each done in duplicate.

Time of incubation (min)	Radioactivity (c.p.m./mg of cellular protein)					
	Phenylalanine			Leucine		
	Control	<i>p</i> -Chloro-phenylalanine	α-Methyl-phenylalanine	Control	<i>p</i> -Chloro-phenylalanine	α-Methyl-phenylalanine
0	18450	—	—	17470	—	—
5	25125	25125	22170	22530	21440	24500
10	23040	19690	24450	24820	24920	23640
15	21790	24380	21570	18980	23740	22925
20	21135	21750	26740	25450	21685	27400

Table 5. *Effects in vivo of p-chlorophenylalanine and α-methylphenylalanine on mouse brain polyribosomes*
Mice were injected with either 2 mg of α-methylphenylalanine/g body wt. or 2 mg of *p*-chlorophenylalanine/g body wt. or an equal volume of 0.9% NaCl and, after 40 min, the brains were removed and homogenized in TKM. Polyribosomes were analysed on a 1.5–0.7M-sucrose gradient as described in the Materials and Methods section. Results are expressed as the ratio of the area under the monoribosomes to the total area. The numbers in parentheses represent the number of independent determinations each done in duplicate. The results are expressed as the means ± s.e.m. for the number of determinations given.

Condition	Ratio monoribosomes/total ribosomes	Increase in mono-ribosomes (%)
Control (3)	0.17 ± 0.02	—
<i>p</i> -Chlorophenylalanine	0.24 ± 0.06	30
α-Methylphenylalanine	0.16 ± 0.005	0

have the same cytotoxic effects during longer incubation periods. In contrast, neither cytotoxicity nor any inhibition of cell growth was observed in cultures incubated with 5mM- or 10mM-α-methylphenylalanine.

p-Chlorophenylalanine increased cell monoribosomes by 40% within 30min, although only about 15% of poly(U)-directed protein synthesis was inhibited by the analogue even when it was present in a 100-fold excess. These results are similar to those of Taub & Johnson (1975), who showed in mouse brain that a 40% increase in cell monoribosomes represented approximately 10% decrease in protein synthesis. These results also indicate that, unlike tyrosine and other large neutral amino acids (Peterson & McKean, 1969; Barra *et al.*, 1973), *p*-chlorophenylalanine did not stoichiometrically block the

incorporation of phenylalanine into protein. *p*-Chlorophenylalanine itself may be incorporated into proteins in place of natural amino acids (Gal & Millard, 1971; Miller *et al.*, 1976). The fact that increasing concentrations of *p*-chlorophenylalanine did not cause a greater decrease in the incorporation of [³H]phenylalanine into polyphenylalanine argues against this. A more sensitive assay to test for this possibility is the aminoacylation *in vitro* of either phenylalanyl- or tyrosyl-tRNA with radioactive amino acids in the presence of *p*-chlorophenylalanine. The inability of the analogue to compete for aminoacylation of either phenylalanyl- or tyrosyl-tRNA showed that *p*-chlorophenylalanine could not be substituted into proteins for either of these amino acids.

Inhibition of the transport of amino acids into neural tissue or cells by high concentrations of another amino acid has been reported for high concentrations of leucine (Roberts & Morelos, 1965), lysine (Richter & Wainer, 1971) and phenylalanine (Agrawal *et al.*, 1970; Vahvelainen & Oja, 1970; Siegel *et al.*, 1971; Oja, 1972). *p*-Chlorophenylalanine inhibited the initial transport of all three of the large neutral amino acids studied. However, *p*-chlorophenylalanine did not affect amino acids already in the cell, in contrast with phenylalanine, which has been shown to cause a rapid efflux of the large neutral amino acids (Hughes & Johnson, 1977*b*). The lack of a significant effect on intracellular amino acid pools by counter-transport indicated that a disrupted cellular supply of amino acids was not responsible for the short-term cytotoxicity observed with *p*-chlorophenylalanine. α-Methylphenylalanine differently affected the initial rate of uptake of each of the three amino acids studied, and did not affect the efflux of either phenylalanine or leucine. Clearly the two analogues do not act at the cell surface in the same manner, which may, in part, explain the difference in cytotoxicity of *p*-chlorophenylalanine

and α -methylphenylalanine. These studies were concerned only with the transport of certain large neural amino acids, and a disruption of ionic transport or flux of other compounds would not have been detected. Alterations in the cellular supply of any component necessary for cellular metabolism could cause inhibition of growth and cell death.

The effects of these analogues *in vivo* paralleled their effects *in vitro*. As had been observed with neuroblastoma cells, *p*-chlorophenylalanine caused a disaggregation of mouse brain polyribosomes similar to that reported for high concentrations of phenylalanine *in vivo* (Aoki & Siegel, 1970; Siegel *et al.*, 1971; MacInnes & Schlesinger, 1971; Taub & Johnson, 1975; Hughes & Johnson, 1977a). Injection of α -methylphenylalanine, on the other hand, had no effect on mouse brain polyribosomes. Although the dose of *p*-chlorophenylalanine used in these studies (2mg/g body wt.) was much larger than the pharmacological dose used to inhibit phenylalanine hydroxylase (Lipton *et al.*, 1967; Antonas & Coulson, 1975; Copenhaver *et al.*, 1973), these results showed that *p*-chlorophenylalanine can disaggregate polyribosomes within a relatively short time. Perhaps the smaller dose used to inhibit phenylalanine hydroxylase would have the same effect after prolonged treatment. An administration of α -methylphenylalanine at the same dose, which was approximately 5 times the concentration used by Greengard *et al.* (1976) to inhibit phenylalanine hydroxylase *in vivo*, did not affect mouse brain polyribosomes *in vivo*. Because this large dose of α -methylphenylalanine failed to disaggregate brain polyribosomes *in vivo*, we doubt that prolonged treatment with the lower dose used to inhibit phenylalanine hydroxylase would disaggregate polyribosomes.

In experimental models using *p*-chlorophenylalanine it would be difficult to distinguish between the toxic effect of the analogue and the effect of the hyperphenylalaninaemia. Because no toxic effects were observed with α -methylphenylalanine, any metabolic and behavioural changes could more likely be attributed solely to hyperphenylalaninaemia when it is used to inhibit phenylalanine hydroxylase.

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