BBA 66232

THE MECHANISM OF INHIBITION OF HYDROXYLASES *IN VIVO* BY *p*-CHLOROPHENYLALANINE: THE EFFECT OF CYCLOHEXIMIDE

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(Received August 17th, 1970)

SUMMARY

p-Chloro-[^{14}C]phenylalanine administered *in vivo* to rats was recovered from acid hydrolysates of purified enzyme protein by amino acid analysis. Inhibition *in vivo* of rat liver phenylalanine 4-hydroxylase (EC 1.14.3.1) was significantly reduced by administration of cycloheximide. The active incorporation of *p*-chloro-[2-^{14}C]-phenylalanine into brain and liver aminoacyl-RNA and protein was also inhibited by cycloheximide. Disc gel electrophoresis patterns of purified phenylalanine 4-hydroxylase are altered following *p*-chlorophenylalanine administration.

INTRODUCTION

Administration of *p*-chlorophenylalanine markedly inhibits phenylalanine 4-hydroxylase (EC 1.14.3.1) of rat liver[1]. Inhibition of cerebral tryptophan 5-hydroxylase *in vivo* was found to be irreversible and the enzyme activity remained low for a period of five to seven days[2]. The irreversible inhibition of rat liver phenylalanine hydroxylase produced by *p*-chlorophenylalanine could be prolonged when followed by administration of puromycin or ethionine[3].

Initial observations revealed that *p*-chlorophenylpyruvic acid was equally as active as *p*-chlorophenylalanine[1]. However, experimental evidence recently obtained indicates that active transamination of *p*-chlorophenylpyruvic acid *in vivo* to *p*-chlorophenylalanine is responsible for its inhibitory effect[4]. In these studies we also reported the appearance of label in various hydroxylases following administration of *p*-chloro-[^{14}C]phenylalanine. The incorporation of *p*-fluorophenylalanine into some enzyme proteins without affecting the enzymic activity has been established[5].

This paper presents evidence that the incorporation of *p*-chlorophenylalanine into the protein is functionally related to the inhibition of phenylalanine 4- and tryptophan 5-hydroxylases.

We propose that the selective inhibition of phenylalanine and tryptophan hydroxylases could be interpreted by incorporation of *p*-chlorophenylalanine at an enzymatic site essential for their activity. Conversely, when incorporation of *p*-chlorophenylalanine is not at a location critical for enzyme activity the enzyme is not inhibited as in the case of tyrosine hydroxylase.

MATERIALS AND METHODS

Male Sprague-Dawley rats (250–350 g) were used throughout. Intracerebral injections were given as described elsewhere[6].

p-Chloro-DL-phenylalanine, *p*-fluoro-DL-phenylalanine and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Mo.; *p*-bromo-DL-phenylalanine from Cyclo Chemical Corp., Los Angeles, Calif.

p-Chloro-DL-[2-^{14}C]phenylalanine ethyl ester hydrochloride was synthesized as described elsewhere[4]. L-[3-^{14}C]-Tryptophan and uniformly ^{14}C-labeled L-tyrosine were purchased from Amersham-Searle Corp., Chicago, Ill.; DL-[3-^{14}C]-*p*-fluorophenylalanine from Calbiochem., Los Angeles, Calif..

Enzyme Assays. Cerebral tryptophan 5-hydroxylase was assayed as previously described[7]. Tyrosine hydroxylase activity was determined according to IKEDA, *et al.*[8]. Liver phenylalanine 4-hydroxylase was assayed following purification through the first $(NH_4)_2SO_4$ fractionation or the AlC_γ gel stage[9]. Elution of enzyme from the AlC_γ gel was slightly modified. We found that after elution of alumina gel with 0.008 M phosphate buffer, elution with 0.1 M potassium phosphate buffer containing 4% $(NH_4)_2SO_4$ at pH 6.8 markedly increased the yield of enzyme protein without affecting its specific activity as reported in the original method[9]. The activity was usually measured with an excess of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine without pteridine reductase[9] and tyrosine determined by the nitrosonaphthol method colorimetrically[10] or spectrofluorimetrically[11] in the assays of hydroxylation of *p*-halophenylalanines *in vitro*. Addition of pteridine reductase, glucose dehydrogenase, NADPH, and glucose leads to an apparent increase in specific activity, without, however, affecting the degree of inhibition by the *p*-halophenylalanines *in vivo*. For kinetic studies the complete system was used. Rat liver phenylalanine 4-hydroxylase (0.44 mg at the AlC_γ stage) from control and *p*-chlorophenylalanine-treated animals was assayed with L-phenylalanine concentrations of 0.5–2.0 mM and 0.4 mM 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine at 37° for 30 min in O_2.

Cerebral tryptophan and tyrosine hydroxylases were purified as described previously[4].

Analytical Procedures. In incorporation studies the brains and livers of animals were removed and the total RNA, soluble RNA and proteins were isolated according to the method of NEMETH, *et al.*[12] with one modification. The proteins obtained following hot $HClO_4$ extraction were washed twice with distilled water and once each with absolute ethanol and ether. The proteins were then dried to constant weight in a 60° oven. Weighed amounts of protein were then dissolved in NCS reagent (Amersham-Searle) for liquid scintillation counting. The absorbancy at 260 nm was determined for the RNA solutions from which the number of milligrams of RNA was calculated using an extinction coefficient of $9 \cdot 10^6$ cm^2/mole (ref. 13).

Samples of the enzyme protein at various stages of purification were applied to a Sephadex G-25 column equilibrated with 0.005 M phosphate buffer, pH 7.4, to separate protein from salts and traces of free amino acids. The fractions containing protein were pooled, an aliquot counted, and the remainder concentrated to dryness *in vacuo* at 35°. The hydrolysate was dried over NaOH pellets. The hydrolysate was taken up in 0.8 ml 10% (v/v) 2-propanol. This solution was submitted to amino acid analysis as described elsewhere[4]. Eluates corresponding to tyrosine, *p*-chlorophenyl-

alanine and *p*-fluorophenylalanine were identified by two-dimensional paper chromatography against authentic samples. The first dimension was run descending in *sec*-butyl alcohol–3% NH_4OH, (150:60, v:v) (R_F *p*-chlorophenylalanine, 0.59; *p*-fluorophenylalanine, 0.64; L-tyrosine, 0.15); the second dimension was developed descending in *sec*-butyl alcohol–formic acid–water (150:30:20, v:v:v) (R_F *p*-chlorophenylalanine, 0.61; *p*-fluorophenylalanine, 0.67; L-tyrosine, 0.38). The spots corresponding to *p*-chlorophenylalanine, *p*-fluorophenylalanine and tyrosine were either developed with ninhydrin or cut out and counted directly by liquid scintillation counting. In the incorporation studies it was necessary to assess how much of the radioactive amino acid was incorporated or adsorbed. The adsorbed radioactivity was removed by the method of CANELLAKIS AND HERBERT[14].

Disc electrophoresis was carried out in polyacrylamide gels, 0.6 cm in diameter and 19 cm in length. The gel mixture consisted of 2 parts 40% acrylamide containing 0.4% *N*,*N*-methylenebisacrylamide, 1 part 0.28% *N*,*N*,*N'*,*N'*-tetramethylenediamine, 1 part 0.2 M Tris–glycine buffer, pH 8.5, and 4 parts 0.2% $(NH_4)_2S_2O_8$. A 1:9 dilution of the 0.2 M Tris–glycine buffer was used as electrolyte. Gels were pre-electrophoresed one hour before the samples were applied. Protein (350–500 μg) was applied in a 10% sucrose solution. Gels were run at 100 V and 10 mA for 18 h. The gels were stained in 0.1% amido blue-black in 7% acetic acid–20% ethanol for 4 h. The electrophoretically destained gels were scanned on a Joyce microdensitometer, Model MK III C using wedge 5519, slit 20, ratio 1:1, and lens 48 mm 0.08.

RESULTS AND DISCUSSION

Earlier experiments[4] indicated conversion *in vivo* of small amounts of *p*-chlorophenylalanine into tyrosine, presumably according to the enzymic conversion observed by KAUFMAN for *p*-fluorophenylalanine and *p*-chlorophenylalanine[15]. We confirm this hydroxylation of *p*-fluorophenylalanine and *p*-chlorophenylalanine *in vitro* by purified hepatic phenylalanine 4-hydroxylase (calcium phosphate gel step). In our experiments, *p*-fluoro-DL-phenylalanine was hydroxylated at 0.25 μmole/mg per h, *p*-chlorophenylalanine 0.015 μmole/mg per h, and *p*-bromo-DL-phenylalanine 0.016 μmole/mg per h while DL-phenylalanine was 0.8 μmole/mg per h.

Experiments with *p*-bromo-, *p*-chloro- and *p*-fluorophenylalanines, aimed at establishing their relative inhibition when administered in equimolar amounts (1.5 mmole/kg), support the findings of KOE AND WEISSMAN[1] on brain 5-hydroxytryptamine levels. In animals sacrificed 24 h after intraperitoneal injection of the above analogues cerebral tryptophan 5-hydroxylase was inhibited 47% by *p*-bromo-, 72% by *p*-chloro- and 12% by *p*-fluorophenylalanine. Of these compounds, only *p*-chlorophenylalanine produced simultaneous inhibition of liver phenylalanine 4-hydroxylase *in vivo*.

The experiments *in vivo* revealed that most of the radioactivity derived from the incorporation of *p*-chlorophenylalanine into enzyme protein. As an extension of these earlier experiments we purified hepatic phenylalanine 4-hydroxylase and cerebral tryptophan 5-hydroxylase from animals receiving intraperitoneal *p*-chloro-[2-^{14}C]phenylalanine or *p*-fluoro-[3-^{14}C]phenylalanine. As a measure of comparison, rats injected with [^{14}C]tyrosine were used as controls. Small portions of the partially

purified enzyme proteins were assayed for enzymic activity. The remainder, following acid hydrolysis, was used for amino acid analyses.

Results (Table I) of these experiments demonstrate that the isolated proteins containing the enzyme activity following removal of adsorbed radioactivity contain measurable amounts of *p*-chlorophenylalanine or *p*-fluorophenylalanine in both phenylalanine and tryptophan hydroxylases. A small amount of label was detectable as [^{14}C]tyrosine in the phenylalanine 4-hydroxylase of *p*-chloro-[^{14}C]phenylalanine-treated animals. In the tryptophan hydroxylase, however, [^{14}C]tyrosine was below the limit of detectability. In a zero-time experiment radioactive *p*-chlorophenylalanine added to liver homogenates of untreated animals incorporated fewer than 12 disint./min per mg protein.

After administration of ^{14}C-labeled *p*-chloro- or *p*-fluorophenylalanine, the ratio of radioactivity incorporated in protein as tyrosine to that as *p*-chloro- or *p*-fluorophenylalanine was 0.31 and 0.49, respectively. This indicates appreciable conversion *in vivo* of *p*-chlorophenylalanine to tyrosine by liver although this is only about half the rate of *p*-fluorophenylalanine hydroxylation under the same conditions. Incidentally, this active hydroxylation of *p*-fluorophenylalanine to tyrosine in the liver accounts for the presence of [^{14}C]tyrosine in proteins from rat pancreas and plasma following administration of *p*-fluoro-[^{14}C]phenylalanine[16].

The incorporation of *p*-chlorophenylalanine or *p*-fluorophenylalanine is not specific for phenylalanine and tryptophan hydroxylases. *p*-Fluorophenylalanine incorporation was demonstrated in several proteins of bacterial and animal cells, albeit without any effect on the enzymic activities of these proteins[5]. One cannot make the assumption from Table I that all the radioactivity is associated with phenylalanine

TABLE I

INCORPORATION OF TYROSINE, *p*-CHLORO- AND *p*-FLUOROPHENYLALANINE IN PARTIALLY PURIFIED HEPATIC PHENYLALANINE 4-HYDROXYLASE AND CEREBRAL TRYPTOPHAN 5-HYDROXYLASE

Two animals for each group were injected with 1.6 mmoles or 90 μC/kg amino acid intraperitoneally and sacrificed 6 h later. Rat liver enzyme was purified through the first $(NH_4)_2SO_4$ step[9]. Part of the enzyme protein was extracted as described in MATERIALS AND METHODS. Tryptophan 5-hydroxylase was prepared as described[4]. Hydrolysate values are corrected for losses due to analytical procedures. p-CP, *p*-chlorophenylalanine; p-FP, *p*-fluorophenylalanine; [U-^{14}C]-L-tyrosine, uniformly ^{14}C-labeled L-tyrosine; [2-^{14}C]-DL-*p*-CP, *p*-chloro-DL-[^{14}C]phenylalanine; [3-^{14}C]-DL-*p*-FP, *p*-fluoro-DL-[^{14}C]phenylalanine.

Enzyme	*Amino acid*	*Enzyme protein radioactivity*		*Acid hydrolysate*		*Tyrosine/p-CP or p-FP*	*Enzyme activity (%)*
		Incorporated (disint./min per mg)	*Adsorbed (disint./min per mg)*	*Tyrosine*	*p-CP or p-FP*	*(disint./min per mg protein)*	
Liver phenylalanine 4-hydroxylase	[U-^{14}C]-L-Tyrosine	549	84	391	0	—	100
	[2-^{14}C]-DL-*p*-CP ethyl ester hydrochloride	78	12	13	42	0.31	85
	[3-^{14}C]-DL-*p*-FP	786	56	236	482	0.49	100
Brain tryptophan 5-hydroxylase	[U-^{14}C]-L-Tyrosine	77	<1	62	0	—	100
	[2-^{14}C]-DL-*p*-CP ethyl ester hydrochloride	27	<1	0	21	—	51

TABLE II

THE *in vivo* INCORPORATION OF *p*-CHLOROPHENYLALANINE AND ITS EFFECT ON CEREBRAL HYDROXYLATION OF TRYPTOPHAN AND TYROSINE

Enzyme assays were done on the 30 000 × *g* supernatants as described in MATERIALS AND METHODS and are averages of duplicate assays on 12 animals. Incorporation studies were done on enzymes purified from the 30% alcohol precipitate of pooled 30 000 × *g* supernatants by sucrose discontinuous density gradient separation[4]. Animals were sacrificed 43 h after administration of *p*-chlorophenylalanine. [^{14}C]*p*-CP, *p*-chloro-[^{14}C]phenylalanine.

Condition	*Tryptophan 5-Hydroxylase*		*Tyrosine Hydroxylase*	
	nmole/mg/h ± *S.E.*	*disint./min per mg enzyme protein*	*nmole/mg/h* ± *S.E.*	*disint./min per mg enzyme protein*
Control	0.40 ± 0.05	—	1.13 ± 0.05	—
[^{14}C]*p*-CP (135 mg ester hydrochloride containing 35 μC)	0.06 ± 0.01	53.5	1.12 ± 0.06	32.8

or tryptophan hydroxylases. Indeed, we find that the protein from brain with tyrosine hydroxylase activity contains, after *p*-chloro-[^{14}C]phenylalanine administration, as much radioactivity as does cerebral tryptophan 5-hydroxylase. The crucial difference is that while the extent of incorporation of the *p*-chloro-[^{14}C]phenylalanine into these enzyme proteins is of the same order of magnitude, the hydroxylation of tyrosine is not inhibited (Table II).

The inhibition of tryptophan 5-hydroxylase in brain is greater at any time than that of hepatic phenylalanine 4-hydroxylase. Nevertheless, this latter enzyme served as the prototype for this study since its purification yields a more homogeneous and active enzyme protein. *p*-chloro-[^{14}C]phenylalanine was present in the enzyme protein at all stages of its purification (94 disint./min per mg at the alcohol stage and 76 disint./min per mg at the AlCγ stage.)

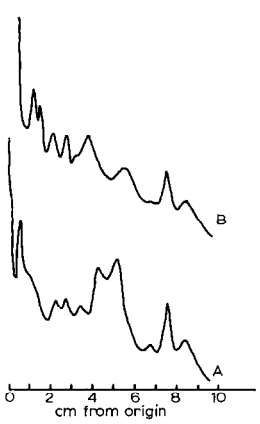

Fig. 1. Microdensitometer traces of phenylalanine 4-hydroxylase (AlCγ stage) after polyacrylamide gel electrophoresis. A: Purified from control rats. B: Purified from *p*-chlorophenylalanine-pretreated rats. Procedure as in MATERIALS AND METHODS.

To determine whether the administration of *p*-chlorophenylalanine to animals would bring about a change in the physical properties of phenylalanine 4-hydroxylase, in five different experiments the enzyme was purified through the AlC_γ gel stage from the livers of untreated and *p*-chlorophenylalanine-treated rats. In each experiment the same amount of protein from *p*-chlorophenylalanine and control samples was developed simultaneously by disc gel electrophoresis. The densitometric tracing of the destained gels demonstrated a consistent difference between the proteins from control and *p*-chlorophenylalanine-treated rat liver (Fig. 1). The double peak at about 5 cm from the origin, invariably present in the control enzyme protein, was replaced in the *p*-chlorophenylalanine protein by a single peak at about 5.5 cm from the origin. The significance of these changes is presently being investigated.

Time-course studies of both enzymic activity and *p*-chloro[^{14}C]phenylalanine incorporation were undertaken to determine a functional relation between the presence of label and the degree of inhibition (Fig. 2). The rapid initial rise in *p*-chloro-[^{14}C]phenylalanine incorporated in the enzyme protein (purified through the AlC_γ step) is concommitant with a rapid drop in enzyme activity. Conversely, the recovery

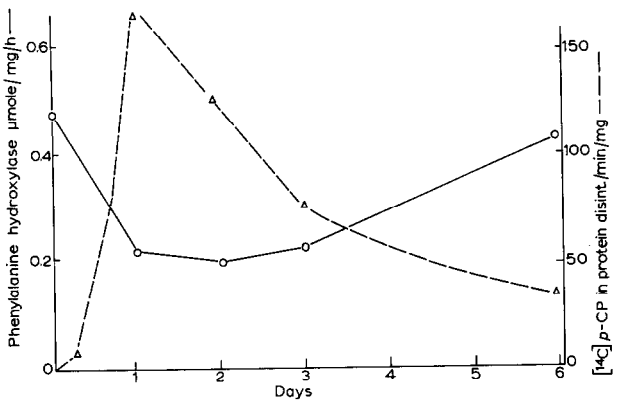

Fig. 2. Phenylalanine hydroxylase activity and *p*-chloro-[^{14}C]phenylalanine ([^{14}C]*p*-CP) in phenylalanine hydroxylase purified to the AlC_γ stage. *p*-chloro-[^{14}C]phenylalanine (300 mg/kg, 90 μC/kg) was administered intraperitoneally at day 0. Enzyme was purified from the combined livers of three rats and assays were run in duplicate.

of enzymic activity to near control values six days later is accompanied by an 80% drop in the radioactivity compared to maximum incorporation. From these data, the half-time for hepatic phenylalanine hydroxylase in the rat is calculated to be 48 h, in agreement with that reported previously[3].

p-Chloro-[^{14}C]phenylalanine was recovered from the hydrolysate of the enzyme protein as described above (Table I). Further experiments established that the presence of *p*-chloro[^{14}C]phenylalanine in the protein was the result of an active process of incorporation rather than adsorption. The results (Table III) reveal that the incorporation of *p*-chloro-[^{14}C]phenylalanine into liver and brain protein is inhibited by cycloheximide in a manner identical to the inhibition of tyrosine incorporation. Although the amount of radioactivity in the free amino acid fraction is different for

TABLE III

EFFECT OF CYCLOHEXIMIDE ON THE INCORPORATION OF *p*-CHLORO-[2-^{14}C]PHENYLALANINE AND [^{14}C]TYROSINE INTO RAT LIVER AND BRAIN PROTEIN

Cycloheximide (2.0 mg) and 2 μC [^{14}C]tyrosine and 1.8 μC *p*-chloro-[^{14}C]phenylalanine were injected intraperitoneally at 0, 1, 2 and 3 h; cycloheximide (400 μg) was also injected into each cortical hemisphere at 0 h. Animals were sacrificed at 4 h. Number of animals in parentheses (first column). *p*-CP, *p*-chlorophenylalanine.

Compounds injected	*Radioactivity recovered*							
	Liver				*Brain*			
	AA (disint./min per mg)*	*RNA–AA** (disint./min per mg RNA)*	*S-RNA–AA*** (disint./min per mg RNA)*	*Protein (disint./min per mg)*	*AA* (disint./min per mg)*	*RNA–AA** (disint./min per mg RNA)*	*S-RNA–AA*** (disint./min per mg RNA)*	*Protein (disint./min per mg)*
Tyrosine (1)	6 940	619	—	209	4 284	913	—	98
Tyrosine + cycloheximide (1)	29 100	230	—	17	11 700	284	—	7
p-CP (2)	42 000	230	14	23	26 250	302	21	16
p-CP + cycloheximide (2)	43 000	93	<1	<1	20 000	104	<1	<1

* Free amino acid (cold $HClO_4$ extract).
** Amino acid esterified to total RNA (hot $HClO_4$ extract).
*** Amino acid esterified to soluble RNA (hot $HClO_4$ extract).

the natural and unnatural amino acids, in the presence of cycloheximide the relative inhibition in the amino acid esterified to the total RNA as well as incorporated in the protein is similar for both amino acids. In one experiment with puromycin aminonucleoside the incorporation of *p*-chloro-[^{14}C]phenylalanine into purified hepatic phenylalanine 4-hydroxylase was reduced by 26%.

In several instances it has been demonstrated that inhibitors of protein synthesis can somehow inhibit catabolism of enzyme protein [17,18]. To test this possibility we followed the effect of cycloheximide on catabolism of labelled protein from brains and livers of animals injected intraperitoneally with L-[^{14}C]tyrosine 24 h prior to administration of 2 mg/kg cycloheximide. For 4 days proteins containing hydroxylase

TABLE IV

EFFECT OF CYCLOHEXIMIDE ON INHIBITION OF HEPATIC PHENYLALANINE 4-HYDROXYLASE BY *p*-CHLOROPHENYLALANINE

Enzyme purified from combined livers of 3 animals; duplicate enzyme assays. *p*-CP, *p*-chlorophenylalanine.

Expt. No.	*Cycloheximide (mg/kg)*	*Time (h)*	*Step of enzyme purification*	*Control*	*Cycloheximide (μmole/mg/h ± S.E.)*	*p-CP*	*p-CP* + Cycloheximide*
1	4	24	$(NH_4)_2SO_4$	0.16 ± 0.01	0.16 ± 0.01	0.08 ± 0.01	0.12**
2	3	24	$(NH_4)_2SO_4$	0.17 ± 0.01	—	0.06 ± 0.01	0.13 ± 0.01
			AlC_γ	0.34 ± 0.02	—	0.05 ± 0.01	0.22 ± 0.02
4	3	48	$(NH_4)_2SO_4$	0.18 ± 0.01	0.16 ± 0.01	0.09 ± 0.01	0.15 ± 0.01
			AlC_γ	0.50 ± 0.01	0.48 ± 0.02	0.20 ± 0.02	0.40 ± 0.01

* *p*-Chlorophenylalanine (300 mg/kg) administered intraperitoneally 2 h after cycloheximide.
** Liver from single animal due to high mortality at 4 mg/kg cycloheximide.

activity were purified from brains and livers of animals daily. At no time was there any difference in label per mg protein between control and cycloheximide animals.

We demonstrated that the incorporation of *p*-chlorophenylalanine is not specific for those enzymes which are inhibited. Hence it is proposed that in tryptophan and phenylalanine hydroxylases *p*-chlorophenylalanine is incorporated near or at the active center of the enzyme. If the inhibition of these enzymes by *p*-chlorophenylalanine is due to its incorporation into their peptidic sequence the inhibition ought to be reduced by inhibitors of protein synthesis. Therefore, cycloheximide was administered to rats 2 h prior to *p*-chlorophenylalanine (Table IV). Cycloheximide significantly decreased *p*-chlorophenylalanine inhibition of phenylalanine 4-hydroxylase. Yet in cummulative doses from 0.6 to 33 mg/kg, even at toxic levels, the hydroxylase activity was not affected. In addition, doses between 2 to 33 mg/kg produced 16–50% mortality within 9 h. These studies on the reversal of *p*-chlorophenylalanine-produced inhibition of phenylalanine 4-hydroxylase by an inhibitor of protein synthesis also established that it is critical that the assays be performed with at least partially purified enzyme. The maximum amount of *p*-chlorophenylalanine found in liver following intraperitoneal administration of 300 mg/kg is 350 μg/g (ref. 4). Therefore, an assay system with 100000 $\times$ *g* supernatant from liver contains from $3\text{–}5 \cdot 10^{-4}$ M *p*-chlorophenylalanine (K', $3 \cdot 10^{-4}$ M)[2,4] over a 48 h period. It is apparent that this concentration of *p*-chlorophenylalanine is enough to inhibit competitively the hydroxylase activity thus masking the reversal of inhibition *in vivo*. Dialysis of the supernatant would remove free *p*-chlorophenylalanine, however, with some loss of enzyme activity[4]. Therefore, purification is the method of choice.

At the present, none of the hydroxylases can be purified to the high degree of homogeneity of some of the crystalline enzymes. This, of course, precludes the presentation of unequivocal proof one may obtain from protein degradation, fingerprinting and sequential peptide analysis. Nevertheless, the mechanism by which inhibition *in vivo* of phenylalanine 4-hydroxylase and tryptophan 5-hydroxylase is brought about by *p*-chlorophenylalanine can logically be explained by the incorporation of this amino acid into these enzymes at a position such as to affect their enzymic activity.

An important corollary to the change brought about by incorporation of *p*-chlorophenylalanine into the enzyme protein was obtained through kinetic studies of phenylalanine 4-hydroxylase purified from the livers of control and *p*-chlorophenylalanine-treated rats. Apparent K_m's as determined from double reciprocal plots were 0.16 mM for control and 1.8 mM for *p*-chlorophenylalanine-containing enzyme. However, v_{max} was 2.0 μmole/mg per h for both samples of enzyme. This would indicate that whatever modification of the enzyme was brought about by the incorporation of *p*-chlorophenylalanine, this did not affect the rate-limiting step of phenylalanine hydroxylation. We infer from the kinetic data and from the changes in the pattern of protein after gel electrophoresis that these changes may be the consequence of the presence of *p*-chlorophenylalanine in the protein with phenylalanine 4-hydroxylase activity.

The following facts are offered in support of this mechanism: (a) there is active incorporation of *p*-chlorophenylalanine into proteins including those with phenylalanine 4-hydroxylase, cerebral tryptophan 5-hydroxylase, and tyrosine hydroxylase activity; (b) during purification of phenylalanine-4-hydroxylase the *p*-chloro-[^{14}C]-

phenylalanine incorporated eventually reaches a nearly constant level of specific activity; (c) the appearance and disappearance of *p*-chloro-[^{14}C]phenylalanine in purified phenylalanine 4-hydroxylase (AlC_γ stage) coincides with the inhibition and recovery of enzymic activity; (d) when incorporation of *p*-chlorophenylalanine into protein containing phenylalanine 4-hydroxylase is prevented by cycloheximide the inhibition is almost completely prevented; and (e) incorporation of *p*-chlorophenylalanine into brain protein leads to concommitant irreversible inhibition of tryptophan 5-hydroxylase without affecting hydroxylation of tyrosine.

The incorporation of monofluorotryptophans into bacterial protein in place of tryptophan causing growth inhibition of wild-type *Escherichia coli* was recently reported[19]. This incorporation apparently yielded proteins with altered properties, thus affecting the growth characteristics of *E. coli*.

Furthermore, the literature on molecular diseases yields additional examples where substitution of a single amino acid for another imparts critical changes in the biochemical and physico–chemical properties of the protein. Therefore, our hypothesis for the mode of action of *p*-chlorophenylalanine is not implausible. This hypothesis also allows for incorporation of *p*-chlorophenylalanine without concommitant irreversible inhibition of other enzyme proteins such as tyrosine hydroxylase.

It should be noted that a degree of competitive inhibition similar to its effect *in vitro* also exists at the early phase (first 72 hours) following administration of *p*-chlorophenylalanine *in vivo*. Since there is also a competitive inhibition of cerebral tyrosine hydroxylase by *p*-chlorophenylalanine *in vitro*[4], such reversible competitive inhibition may account for recent reports of a 30% decrease in cerebral norepinephrine in *p*-chlorophenylalanine-treated rats[20] during the first 72 h.

It now appears that the mechanism of inhibition *in vivo* of some hydroxylases by *p*-chlorophenylalanine could be explained (a) primarily by its incorporation into enzyme protein near or at the active site; (b) by reduction of transport of some natural amino acids[4]; and (c) possibly to some extent by a degree of competitive inhibition similar to its mechanism *in vitro*[2,4] soon after its administration *in vivo*.

ACKNOWLEDGEMENTS

The skillful technical assistance of Phyllis K. Peterson and Murray Bouschlicher is gratefully acknowledged. The authors thank Professors R. L. Blakley, G. R. Chalkley, and T. W. Conway, Department of Biochemistry, University of Iowa, for their discussions of kinetics, electrophoresis techniques, and antibiotic action, respectively.

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