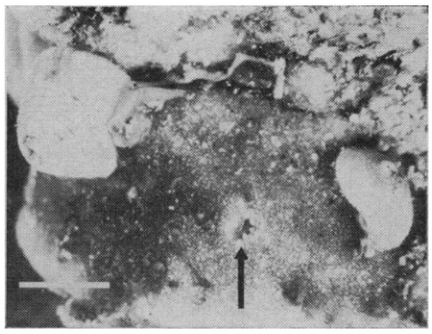

Fig. 1. Pair of live *Heliacus trochoides* on under surface of *Palythoa tuberculosa* colony, with hole where one has fed (arrow). North Male Atoll, Maldive Islands. Scale, 3 mm.

When *in situ* the animals are in various positions, including umbilical side up. In the laboratory, feeding and spawning occurred only at night, and no direct observations were made of either activity. However, *Heliacus* does eat *Palythoa* body tissues; 3- to 4-mm deep holes were made overnight in the surface of the colonies (Fig. 1, arrow). Also, the feces of *Heliacus*, which are uncompacted, are the same pale yellowish brown color as *Palythoa* and contain sand grains derived from the sand-containing *Palythoa* tissues. The spawn of *H. cylindricus* is a U-shaped jelly mass that is attached with mucous threads to the host colony.

This case of symbiosis I consider to be ectoparasitism verging on commensalism. *Heliacus* does not harm its host much (the injured tissues heal speedily), and, relative to a host colony, even a full-grown *Heliacus* is small [rarely larger than a single zoanthid polyp (*15*)].

The larval ecology of *Heliacus* is noteworthy because there is at least occasional transoceanic larval transport by ocean currents. Architectonicid larvae can live in the plankton more than 3 months (possibly 7 months or more), and the West Indian architectonicids (including several species of *Heliacus*) occur in shallow water across the entire tropical Atlantic (*16*). The observations in this paper imply that contact with benthic zoanthiniarians is essential for successful settlement and metamorphosis of these *Heliacus* larvae and for their subsequent growth and attainment of sexual maturity.

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References and Notes

1. Architectonicidae and *Heliacus* are also known under the names Solariidae and *Torinia*. Bayer (*2*) lists 54 Recent species of *Torinia*; my estimate is based on his list but excludes synonyms and species in other genera.
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14. The species identifications of the zoanthids are provisional because the taxonomy is in disorder (according to C. E. Cutress, who identified them). The catalog numbers of the host specimens at the United States National Museum (U.S.N.M.), Washington, D.C., are listed to aid eventual revision.
15. *Heliacus bicanaliculatus* is exceptional in attaining the volume of 10 to 15 polyps of *Zoanthus danai*.
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17. My field work was supported by various organizations: in the Bahamas, by the Society of the Sigma Xi and the Lerner Marine Laboratory of the American Museum of Natural History; in Florida, by the Marine Laboratory, University of Miami; in British Honduras, by the Humble Oil and Refining Company in a grant to E. G. Purdy (Rice University); and, in the Maldives, by the National Science Foundation as a part of the U.S. Program in Biology, International Indian Ocean Expedition. For the field observations and specimens from the Hawaiian Islands, I thank Mrs. E. Gage, Mrs. A. M. Harrison, and A. E. Kay; from the Gulf of California, D. R. Shasky; from Aruba, R. T. and S. D. Abbott, and Mrs. J. O. Johnson. For other information, I thank C. E. Cutress, A. M. Keen, T. L. McGinty, A. S. Merrill, and R. S. Scheltema.

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p-Chlorophenylalanine-Induced Chemical Manifestations of Phenylketonuria in Rats

Abstract. p-*Chlorophenylalanine, a potent inhibitor of phenylalanine hydroxylase in vivo, has been used to simulate phenylketonuria in rats. This inhibitor, when administered with phenylalanine, produces marked elevation of blood and tissue phenylalanine without an increase in tyrosine. Disproportionate ratios of phenylalanine to tyrosine are characteristic of phenylketonuria in humans. The use of* p-*chlorophenylalanine permits the production of this characteristic amino acid imbalance in experimental animals.*

Human phenylketonuria (PKU) is characterized by a genetically linked deficiency of phenylalanine hydroxylase in the liver (*1*). The immediate consequence of this deficiency is the inability of the subject to convert ingested phenylalanine to tyrosine. This is reflected in a markedly increased content of phenylalanine in the blood (*2*) with normal or slightly diminished amounts of tyrosine (*3*). Other consequences of this metabolic defect, such as the elevation of phenylpyruvic acid in blood and urine (*4*) and the lowering of platelet serotonin and urinary 5-hydroxyindole acetic acid (*5*), are apparently secondary to the primary defect.

The need for an experimental model of PKU for metabolic and behavioral studies in laboratory animals is obvious. However, the production of the condition in animals has met with only limited success. Two techniques have been used: (i) the inclusion of phenylalanine (5 to 7 percent) in the diet (*6*), and (ii) the use of a genetic strain of mice with decreased phenylalanine hydroxylase activity (*7*). More recently it has been shown that hamsters have a much lower level of phenylalanine hydroxylase activity than rats have (*8*) and develop hyperphenylalaninemia when fed diets containing excess phenylalanine [for a detailed review of the various methods see (*9*)].

Although these methods lead to conditions resembling human PKU in that they produce hyperphenylalaninemia, abnormal phenylalanine tolerance curves, and some of the appropri-

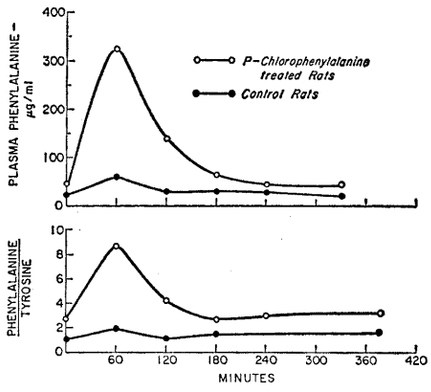

Fig. 1. Two male rats were given 300 mg of *p*-chlorophenylalanine (*p*-CPA) per kilogram of body weight and then were given a second dose of 100 mg/kg 24 hours later. L-Phenylalanine (200 mg/kg) was administered to these animals and to two control animals 24 hours after the second dose of *p*-CPA. Blood was drawn from the tail vein at the intervals shown above and assayed for phenylalanine and tyrosine. Each point represents the average obtained on samples from two animals.

ate aromatic urinary metabolites, they fail in at least one important respect to produce the primary defect of the human condition, that is, inhibition of liver phenylalanine hydroxylase which is manifested by elevated amounts of phenylalanine and normal amounts of tyrosine in the blood. Because, in the experimental models, phenylalanine hydroxylase is, at best, only partially inhibited, concentrations of tyrosine in blood and tissues are elevated to almost the same extent as phenylalanine. Since tyrosine levels are not increased in PKU, the models cannot be used to investigate the many important consequences of the markedly disproportionate concentrations of the two amino acids which exist in the clinical disorder.

Recently, Koe and Weissman (*10*) demonstrated that administration in vivo of *p*-chlorophenylalanine (*p*-CPA) strongly inhibited both tryptophan and phenylalanine hydroxylase activity of rat liver and markedly diminished the amount of serotonin in the tissues. Their results prompted this investigation, the primary aim of which was to determine to what extent treatment with *p*-CPA could inhibit hydroxylation of phenylalanine in the rat in vivo and to what degree this experimental condition resembled the genetic condition, PKU, in man.

Combined phenylalanine and *p*-CPA were assayed by the method of McCaman and Robins (*11*), and tyrosine by the method of Waalkes and Udenfriend (*12*). Phenylalanine was dissolved in water, adjusted to *p*H 3.5, warmed to 40°C, and injected intraperitoneally. *p*-Chlorophenylalanine was suspended in saline and also administered intraperitoneally. Male Sprague-Dawley rats (about 200 g) were used; they were fasted overnight before each experiment involving measurement of blood or tissue concentrations. This was done to minimize fluctuations in plasma amino acid levels due to dietary protein. Liver phenylalanine hydroxylase was assayed by an isotopic method (*13*). Separation and quantitation of *p*-CPA and phenylalanine in tissue extracts were performed on the short column of the amino acid analyzer at *p*H 5.2 and 50°C (*14*).

Rats were given 300 mg of *p*-CPA per kilogram of body weight, and a second dose of 100 mg per kilogram was given 72 hours later. They were killed 24 hours after the second dose, and the plasma was assayed for phenylalanine, tyrosine, and *p*-CPA. The plasma phenylalanine concentrations were increased (25 to 75 μg/ml) compared to control values (10 to 15 μg/ml). Concentrations of tyrosine were slightly lower in the animals treated with *p*-CPA (9 μg/ml) than in the controls (12 μg/ml). Appreciable amounts of *p*-CPA, usually of the same order of magnitude as phenylalanine, were also present in the plasma.

When rats were treated with *p*-CPA in a similar manner and then given a loading dose of phenylalanine (50 mg/kg), there was a marked contrast between the phenylalanine tolerance curves of the treated animals and those of control animals (Fig. 1). In these studies, ratios of phenylalanine to tyrosine as high as 8.5 : 1 were achieved in the animals treated with *p*-CPA. This may be contrasted with the maximum ratios of 2 : 1 obtained in similar tolerance tests with normal animals. In another experiment in which *p*-CPA (300 mg/kg) was administered and the animals were tested for phenylalanine tolerance 24 hours later, initial phenylalanine levels of 75 μg per milliliter of plasma were obtained, and ratios of phenylalanine to tyrosine of 20 : 1 were achieved. Apparently, the drug effectively blocks phenylalanine hydroxylase in vivo; this is reflected by the abnormally high phenylalanine tolerance curve and the failure to convert phenylalanine to tyrosine.

Table 1. Amounts of phenylalanine (Phe) and tyrosine (Tyr) in plasma and brain of rats treated with *p*-chlorophenylalanine. Eight rats were given a single dose of *p*-CPA (300 mg/kg); 24 hours later four of them were started on a regimen of phenylalanine (50 mg/kg) every 4 hours for 24 hours, and the remaining four were used as controls. Four hours after the last dose of phenylalanine all the animals were killed, and plasma and brain were assayed. All injections were given intraperitoneally, and diluent alone was injected into all control animals. The results are expressed as the average obtained on four animals plus or minus the standard error.

Amino acid	Plasma (μg/ml)	Brain (μg/g)
Rats treated with p-*CPA*		
Phenylalanine	63.0 ± 5.9	37.0 ± 7.4
Tyrosine	8.4 ± .4	11.2 ± .4
Phe/Tyr	7.5	3.3
Rats treated with p-*CPA and phenylalanine*		
Phenylalanine	143.0 ± 27.0	75.0 ± 17.8
Tyrosine	10.6 ± .5	13.0 ± .7
Phe/Tyr	13.5	5.8

To determine the effect of prior treatment with *p*-CPA together with phenylalanine loading upon the content of phenylalanine and tyrosine in the tissues, eight rats were given 300 mg of the drug per kilogram of body weight intraperitoneally. Three days later, four of the rats were given repeated injections of phenylalanine (50 mg/kg) at 4-hour intervals for 20 hours and were killed 4 hours later. The other four rats were untreated during this period. Blood and brain were analyzed for phenylalanine and tyrosine. The phenylalanine content of the tissues was increased after administration of *p*-CPA alone and became even higher in those animals that were also given phenylalanine (Table 1). The high content of phenylalanine in the tissues was achieved without a corresponding rise in tyrosine. Consequently, the ratio of phenylalanine to tyrosine was raised severalfold above that found in normal tissues.

Assays of liver phenylalanine hydroxylase (Table 2) revealed a marked inhibition in animals treated with *p*-CPA. Dialysis of the liver preparations from the animals treated with *p*-CPA did not restore the enzyme activity. Only slight inhibition could be observed when *p*-CPA was added to either crude or purified enzyme from normal animals, even in concentrations of $8 \times 10^{-3} M$. The amount of *p*-CPA in the livers of treated animals in these experiments was estimated as less than 0.2 μmole per gram, or less than $2.8 \times 10^{-6} M$ in the incubation mixture.

None of the observed products of the action of phenylalanine hydroxylase on *p*-CPA, tyrosine, *m*-chlorotyrosine, or *p*-chloro-*m*-tyrosine (*14*) had significant inhibitory effects in vitro. The long-lasting inhibition of phenylalanine hydroxylase in vivo is apparently neither competitive with substrate nor readily reversible. The mechanism of this apparently irreversible inhibition is as yet unknown but remains of great interest.

Our studies on the ability of *p*-CPA to inhibit liver phenylalanine hydroxylase in vivo corroborate and extend the findings of Koe and Weissman (*10*).

Table 2. Effect of *p*-chlorophenylalanine on rat liver phenylalanine hydroxylase. Crude liver extracts were prepared by homogenizing the tissues with two volumes of 0.01*M* acetic acid and centrifuging. The supernatant was assayed in 0.25-ml incubations containing extract, 5 or 10 μl; L-phenylalanine, 1 μmole; *p*-tritio-L-phenylalanine, approximately 200,000 count/min; reduced nicotinamide adenine dinucleotide (NADH), 1 μmole; 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, 0.15 μmole (in 0.03 ml of 0.1*M* mercaptoethanol); and tris buffer, *p*H 7.3, 25 μmole. The assay mixtures were incubated for 10 minutes at 30°C, and then the tubes were boiled for 1 minute. Water (0.5 ml) and *N*-iodosuccinimide (0.2 ml of a 1 percent solution) were added, and the tubes were held at 0°C for 5 minutes. Then 0.05 ml of 30 percent trichloroacetic acid was added, and the contents were passed over short Dowex-50 columns. The tritiated water eluted from the column was a measure of the hydroxylase activity (*13*). Small samples of liver extracts were dialyzed overnight at 0°C in 15 liters of 0.05*M* tris, *p*H 7.3. Experiments were done in vitro with crude extracts from control animals.

Sample	Activity (%)
Control	100*
Control, dialyzed	103
p-CPA in vivo	19
p-CPA in vivo, dialyzed	20
p-CPA in vitro ($8 \times 10^{-3}M$)	82

* One hundred percent activity represented 4724 count/min from 10-μl samples (average of four animals; range, 3990 to 5810 count/min), with a blank of 1058 count/min. The *p*-CPA samples gave an average of 1755 count/min (range, 1437 to 2110 count/min).

Table 3. Serum phenylalanine to tyrosine (Phe/Tyr) ratios in clinical and in experimental phenylketonurias.

Subjects	Phe/Tyr ratio
Previous studies	
Normal humans	Approx. 1
Phenylketonuric patients (*3, 22*)	Range, 15 to 50
Normal rats (*6*)	1.2
Rats on high-phenylalanine diet (*6*)	1.1
Present study	
Normals rats	1.2
Rats treated with *p*-CPA and phenylalanine	Range, 8 to 20

Thus *p*-CPA can be used to simulate the biochemical lesion of human PKU. It is generally assumed that most of the pathology associated with PKU is due to excess phenylalanine or its metabolites which accumulate, or both. One possibility is that the abnormally large amounts of phenylalanine interfere with tyrosine by competing with the latter for specific enzymes. Phenylalanine or its metabolites, or both, inhibit the conversion of tyrosine to melanin (*15*) and to norepinephrine (*16*), and may also interfere with the oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid (*17*). In addition, phenylalanine may inhibit the uptake of tyrosine or other amino acids into the brain (*18*). In the experimental models of PKU, which have been used, the concentrations of both phenylalanine and tyrosine were increased so that the effects of phenylalanine were either minimized or totally offset by the increased tyrosine. A summary of the plasma phenylalanine to tyrosine ratios observed in human PKU, in phenylalanine-fed rats, and in rats treated with *p*-CPA is given in Table 3. Although values of phenylalanine in the blood of mice bred for low phenylalanine hydroxylase have not been reported, it has been shown that the tyrosine levels in the blood of such mice rise appreciably after phenylalanine loading (*7*). Thus, it can be seen that only in animals treated with *p*-CPA does the tyrosine content of the blood remain low even though the phenylalanine concentration is markedly elevated, an essential criterion of PKU.

The use of *p*-CPA should make it possible to investigate the effects of abnormally high levels of phenylalanine on tyrosine hydroxylase, the rate-limiting step in norepinephrine biosynthesis. Phenylalanine has been shown to inhibit the conversion of tyrosine to 3,4-dihydroxyphenylalanine by purified tyrosine hydroxylase (*19*). Like other tyrosine hydroxylase inhibitors such as α-methyltyrosine (*20*), it may therefore block norepinephrine formation in vivo. Studies with rats treated with *p*-CPA have shown that norepinephrine concentrations are lowered in some tissues in response to administered phenylalanine.

Since the highest plasma phenylalanine levels were obtained after a phenylalanine load, it is probable that the best way to use *p*-CPA experimentally would be in conjunction with phenylalanine in the diet. It may be possible to use a high-protein diet as the source of phenylalanine, thereby simplifying animal investigations in this field.

It should be noted that *p*-CPA is not an inhibitor of liver phenylalanine hydroxylase alone but also inhibits tryptophan-5-hydroxylase in brain and other tissues (*21*). Although there is evidence that serotonin levels are diminished in PKU (*5*), they are not lowered as markedly as in animals treated with *p*-CPA at this dosage (*10*). Thus animals treated with *p*-CPA may be useful for investigating phenylalanine-tyrosine interrelationships in PKU, but they may provide misleading information with respect to 5-hydroxyindole metabolism in this disorder.

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