Developmental Pattern of Phenylalanine Hydroxylase Activity in the Chicken


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ABSTRACT Experiments were conducted to determine the conditions for assay of hepatic phenylalanine hydroxylase (PAH) activity in the chicken and to determine the developmental pattern of PAH activity in liver 25,000 × g supernatant. PAH activity was detected in liver supernatant and (postnuclear) 25,000 × g particulate fraction. Optimum assay conditions differed for the two cell fractions, the most notable difference being a broad pH optimum of 7.7 to 9.2 for the supernatant and 4.7 and 5.6 for the particulate fraction. The PAH activity in the supernatant increased to a maximum as L-phenylalanine concentration in the assay medium increased from 0.02 to 0.5 mM and 1.0 mM. Activity increased in the particulate fraction as the Phe concentration increased to 0.5 mM. Substrate inhibition of PAH activity occurred at Phe concentrations of 3 to 5 mM in the supernatant but not in the particulate fraction. Concentrations of the cofactor, 6(R)-5,6,7,8-tetrahydrobiopterin, ranging from 0.09 to 0.75 mM, resulted in maximal PAH activity. The developmental pattern of PAH in supernatant was determined using a modified assay in which substrate and cofactor concentrations and pH were optimum. The PAH activity in liver supernatant was present at a low level in 11 d chick embryos and increased several fold between Days 15 and 17 to a maximum at Days 17 to 21. Activity declined at hatching to levels that were present in 11 to 15 d embryos and remained at this level in male chicks through 4 wk of age. Mature males had higher PAH activity than mature laying females.

(Key words: L-phenylalanine, hydroxylase, biopterin, pH)

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INTRODUCTION Phenylalanine hydroxylase (PAH), the limiting enzyme of Phe metabolism, catalyzes the conversion of L-Phe to L-Tyr. Liver is the main site of PAH activity, although some activity has been observed in kidney in various mammalian species that have been investigated (Udenfriend and Cooper, 1952; McGee et al., 1972; Murthy and Berry, 1975; Rao and Kaufman, 1986). Developmental patterns of PAH activity in mammals have been described: the most complete information for an extended period of development has been generated using the laboratory rat (Reem and Kretchmer, 1957; Freedland et al., 1962; Friedman and Kaufman, 1971; McGee et al., 1972; Tourian et al., 1972; Barranger, 1976; Dhondt et al., 1979; Yeoh et al., 1988) and guinea pig (Berry et al., 1972). Avian species also are of interest because the embryo develops in an environment isolated from maternal metabolism and the chick is precocious at the time of hatch. Therefore, it is possible to study the need for, or effects of, Phe or its metabolites during embryonic development or in the neonatal period without interference from concurrent maternal metabolism.

Brenneman and Kaufman (1965) observed PAH activities in livers of special species, including newly hatched and adult chickens. Strittmatter and Oakley (1966) assessed the developmental pattern of PAH activity in the chicken using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate to regenerate the natural reduced pteridine cofactor, tetrahydrobiopterin, for PAH activity using assay conditions that had been developed for rat liver. They observed that low activity of PAH could be detected in liver at 12 d of incubation and that the activity increased markedly by 18 d, declining slightly after hatching at 20 d, but generally persisting at the increased level through 8 d posthatching. The activity pattern was similar, although total activity was lower, in 15,000 × g and 100,000 × g supernatants as compared to the pattern of activity in crude homogenates of liver.

The present study was conducted to consider the activity of PAH using reduced tetrahydrobiopterin cofactor and optimal conditions for assay in the chicken and to extend the period of investigation from 11 d of

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Abbreviation Key: NADPH = reduced nicotinamide adenine dinucleotide phosphate; PAH = phenylalanine hydroxylase.
incubation to 4 wk after hatching. Mature males and females were assayed for comparative purposes.

MATERIALS AND METHODS

Animals

All chickens and fertile eggs were obtained from the Cornell K-strain (Cole and Hutt, 1973) of Single Comb White Leghorn chickens maintained at the University’s Poultry Research Farm. Eggs were incubated at 37.2 C at 84% relative humidity until 18 d of incubation, after which time they were incubated at 36.9 C and 87% relative humidity. Chicks had free access to a chick starter feed and mature chickens had free access to a layer-breeder feed. Drinking water was continuously available. Chickens were subdued with CO2 gas and euthanatized by cervical dislocation prior to tissue sampling according to procedures approved by the Institutional Committee on Animal Care and Use. Embryos were euthanatized by decapitation before tissue sampling.

Tissue Preparation

Liver samples were blotted dry and immediately put on ice. In order to obtain a representative sample, the fresh livers were pooled, chopped with scissors, and minced. Approximately 2.5 g of pooled liver was used for each experiment. Liver samples were then homogenized with motor-driven Teflon® pestles (990 rpm) in a Potter-Elvehjem homogenizer using one part liver to nine parts 0.15 M KCl. Five passes were made through tissue with a Teflon® pestle to smooth glass clearance of 0.66 mm followed by five passes with a pestle to glass clearance of 0.30 mm. The homogenate was centrifuged at 460 × g for 15 min in a Beckman model J-21B centrifuge using a JA-20 rotor. The pellet was discarded and the supernatant was centrifuged again at 25,000 × g for 10 min. The supernatant was used for assay of PAH activity in Experiments 1 through 7. Characteristics of PAH activity in the 25,000 × g pellet also were investigated in Experiments 1 through 5. The crude particulate fraction was rinsed once with 0.15 M KCl and recentrifuged at 25,000 × g for 10 min. The pellet was then resuspended, using the Potter-Elvehjem homogenizer, in 4.0 mL of 0.15 M KCl and subjected to three cycles of freezing at −20 C and thawing to disrupt membranes prior to PAH assay. The supernatants and particulate fractions were kept on ice at all times.

PAH Assay

The assay of McGee et al. (1972) was used as the basis from which to optimize assay conditions for the chicken. The assay of McGee et al. (1972) is carried out in 1.0 mL volume in 100 mM potassium phosphate buffer (pH 6.8), 10 mM L-Phe and 0.75 mM 6,7-dimethyl-5,6,7,8-tetrahydropterin (synthetic cofactor), and 5.0 mM dithiothreitol at 35 C for 20 min. The only modification from McGee et al. (1972) initially was incubation at 35 C instead of 25 C and substitution of the natural cofactor, 6(R)-5,6,7,8-tetrahydrobiopterin, for the synthetic cofactor. Each reaction tube contained 1.3 to 2.3 mg protein from supernatant or particulate fraction. Based on the results of the present experiments, the assay was further modified as described in the Discussion. Substrate blanks were used in all assays except in Experiments 3 through 5. Tyrosine was determined by the method of Udenfriend and Cooper (1952) as modified by Nielsen (1969). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Three experiments were carried out to determine the optimum conditions for assay of PAH in the chicken. Substrate concentration was varied from 0 to 5.0 mM in two experiments (Experiments 1 and 2), cofactor concentrations varied from 0 to 0.75 mM (Experiment 3), and the pH of the assay medium was varied from 6.2 to 7.7 in Experiment 4 and from 7.7 to 9.2 for supernatant and 4.7 to 6.2 for particulate fraction in Experiment 5. Livers for these experiments were obtained from 4- or 5-wk-old chicks. Two subsequent experiments (Experiments 6 and 7) were carried out to determine the development pattern of PAH in liver supernatant using assay conditions that were determined as optimal in the initial experiments. Experiment 6 involved embryos from eggs incubated 11 to 21 d. Experiment 7 involved embryos from eggs incubated 11 to 21 d, male chicks at 1 wk and 4 wk of age, and mature males and egg-laying females at approximately 14 mo of age. All livers in Experiments 6 and 7 were collected on the same day and analyzed simultaneously for PAH activity.

Statistical Analyses

Data are means ± SEM. Differences among means were determined by Tukey’s HSD test using Minitab software.

RESULTS

The relationship between Phe concentration and the formation of Tyr in supernatant and particulate fraction is presented in Figure 1. There was little PAH activity at L-Phe concentrations of 0.1 mM or less. Activity was
maximal in the supernatant at 0.5 to 1.0 mM Phe and declined \( (P < 0.05) \) at 3.0 and 5.0 mM Phe. Activity in the particulate fraction was consistently about 40% higher when the fraction was frozen at -20°C and thawed three times than when the fraction had not been frozen: the activities in both particulate preparations increased \( (P < 0.05) \) as the Phe concentration of the incubation medium increased. There was no significant differences \( (P > 0.05) \) in PAH activity among the four highest concentrations of Phe in either particulate fraction. The activities of unfrozen and frozen and thawed particulate fractions were 13 and 18%, respectively, of the activity in supernatant when substrate concentration was 0.5 to 1.0 mM.

The concentration of cofactor, 6(R)-5,6,7,8-tetrahydrobiopterin, was varied from 0 to 0.75 mM using assay conditions of McGee et al. (1972) at 35°C except for substrate concentration which was reduced from 10 to 0.5 mM. Under these conditions the activity of PAH within supernatant and particulate fractions did not differ \( (P > 0.05) \) as the cofactor concentration was varied from 0.09 to 0.75 mM (Figure 2). There was no PAH activity in the absence of cofactor. In the presence of cofactor, the PAH activity in particulate fraction was approximately 12.5% of the activity in the supernatant.

The effect of pH on PAH activity differed markedly between the supernatant and the frozen and thawed particulate fraction (Figure 3). The PAH activity in supernatant increased \( (P < 0.05) \) as the pH was increased from 6.2 to 7.7 but did not change \( (P > 0.05) \) as the pH was increased from 7.7 to 9.2. In the particulate fraction, PAH activity was maximal at pH values in the range of 4.7 to 5.6. Activity in this fraction decreased \( (P < 0.05) \) when the pH of the incubation mixture was raised from 5.6 to 5.9 or 6.2. The mean activity of supernatant from pH 7.7 to 9.2 was 112 nmol of Tyr formed/mg protein per 20 min, whereas the mean activity of particulate fraction from pH 4.7 to 5.6 was 68 nmol of Tyr/mg protein per 20 min.

The results of two experiments to evaluate the developmental pattern of PAH activity in supernatant
are shown in Figure 4. PAH activity was present as early as 11 d of incubation, but increased three- to fourfold by 17 d (Figures 4A and 4B). It remained at this higher level until hatching, at which time it declined to levels that were not significantly different (P > 0.05) from the activities in embryos prior to 17 d of incubation (Figure 4B). One- and 4-wk-old male chicks did not differ significantly (P > 0.05) in PAH activity: mature males had higher (P < 0.05) PAH activity than laying females (Figure 4B).

**DISCUSSION**

Previous assays of PAH have been conducted under various conditions of substrate concentration, cofactor, and pH. Many, including the assays in the previous studies of the chicken (Brenneman and Kaufman, 1965; Strittmatter and Oakley, 1966), have utilized Phe concentrations of 2 mM or higher (Reem and Kretchmer, 1957; Freedland et al., 1962; Nielsen, 1969; Friedman and Kaufman, 1971; McGee et al., 1972; Tourian et al., 1972; Murthy and Berry, 1975; Barranger, 1976; Yeoh et al., 1988). Some have used Phe concentrations of 1 mM or lower (Dhondt et al., 1979; Rao and Kaufman, 1986; Parniak et al., 1988) but all, including those using higher Phe concentrations, involved assays at pH 6.7 to 7.0. The results of the present studies are consistent with the early report of Udenfriend and Cooper (1952), using a rat liver supernatant preparation and natural cofactor, that Phe concentrations greater than 1 mM were inhibitory to PAH activity. Udenfriend and Cooper (1952) stated that the rate of Tyr formation was maximal at pH 7. More recent studies of PAH in the rat indicate that the pH optimum for purified rat liver PAH using synthetic cofactor is near 7.0, whereas the optimum using natural cofactor is maximal near 8.5 (Parniak et al., 1988). The present studies indicate that chicken liver supernatant incubated with natural cofactor has a broad alkaline pH optimum, similar to purified rat liver PAH incubated with natural cofactor. Based on the activity of PAH in supernatant in response to substrate (Figure 1), cofactor (Figure 2), and pH (Figure 3), the PAH assay was modified to provide Phe and 6(R)-5,6,7,8-tetrahydrobiopterin concentrations of 0.5 and 0.375 mM, respectively, and to carry out the assay at pH 8.2 in the study of the developmental pattern of liver PAH activity.

The particulate fraction, which can be expected to contain mitochondria, peroxisomes, and lysosomes, exhibited significant conversion of Phe to Tyr as determined by the PAH assay. At optimum pH (pH 4.7 to 5.6), the accumulation of Tyr was 60% of the accumulation in supernatants incubated at optimal pH (pH 7.7 to 9.2). Strittmatter and Oakley (1966) noted that some PAH activity in supernatant was lost during each centrifugation when homogenates of chicken liver were centrifuged at 15,000 × g and when the resultant supernatant was centrifuged at 100,000 × g. They suggested that some activity may have been associated with the particulate fraction. PAH activity apparently has not been detected in the particulate fraction in other species. The developmental pattern of the PAH activity in the particulate fraction in the present study was not investigated because more research is needed to confirm the existence of actual PAH activity and to determine the subcellular organelle with which it is associated.

Some investigators report that Tyr forms nonenzymatically under some assay conditions (Bobst and Viscontini, 1966; Woof et al., 1971; Abita et al., 1974). Tyrosine was not produced by supernatant or particulate fractions of chicken liver when the fractions in complete reaction mixtures had been pretreated by boiling prior to assay. Substrate blanks and complete reaction mixtures containing boiled supernatant or particulate fraction did not differ in Tyr content (data...
not shown). Therefore, the PAH assay under conditions determined to be optimal for the chicken is not compromised by spontaneous formation of Tyr.

The pattern of PAH during embryonic development is similar to that observed by Strittmatter and Oakley (1966). Activity was present in liver at 11 d of incubation, but it remained at a relatively low level until approximately 16 d and increased to a maximum from 17 d of incubation until near the time of hatching. Previous studies (Austic and Grau, 1971) indicated that no $^{14}$C-Tyr was detected in proteins of 5 and 6 d chick embryos that had been incubated in ovo in the presence of $^{14}$C-Phe beginning at 4 d. A similar lack of conversion was observed in 5 d Japanese quail embryos that had been exposed to $^{14}$C-Phe since the beginning of incubation. A 5 d Japanese quail embryo is morphologically similar to a 6 to 6.5 d chick embryo (Padgett and Ivey, 1960). Tyrosine is the precursor of melanin pigment. Therefore, the lack of conversion of $^{14}$C-Phe to $^{14}$C-Tyr was consistent with the failure of the eyes of chick embryos from 4 to 6 d of incubation to become pigmented when cultured in ovo using a chemically defined medium that contained Phe but no Tyr (Grau et al., 1965; Austic and Grau, 1971). We conclude that the chick embryo develops PAH activity between 6 and 11 d of incubation.

PAH activity declined rapidly at the time of hatching, and remained low through 4 wk of age. Chicks hatched slightly later in Experiment 6 than in Experiment 7, their rate of development being slower for reasons that are not known. Most of the chicks in Experiment 7 at 21 d of incubation, for example, that were used for PAH assay had pipped; however, none had pipped in Experiment 6. This difference in rate of development may explain the slightly earlier increase in PAH activity during the 2nd wk of incubation, and the tendency for PAH activity to decrease at 21 d in Experiment 7 as compared to Experiment 6.

The pattern of PAH development after hatching is different from that observed by Strittmatter and Oakley (1966), who reported a slight decline in activity after hatching but recovery to prehatch levels by Day 4 to 8 of age. These investigators compared activities of liver homogenates and post-mitochondrial fraction and post-microsomal supernatants. The two supernatants had progressively less activity (per gram of liver) than the homogenate, but the developmental patterns were parallel. The lower activity of supernatants as compared to homogenate suggests that the particulate fraction or particulate activity may have existed but it followed a similar developmental pattern of activity as cytosolic PAH. The reason for the difference in PAH activity after hatching in the present study and that of Strittmatter and Oakley (1966) is not clear. The present investigation involved Leghorn chicks, whereas Strittmatter and Oakley (1966) used chicks of a Leghorn-Vantress cross. It is possible that differences in genetic background or environmental conditions may have contributed to the differing patterns of PAH development.

Mature males had higher PAH activity than mature females. This difference is similar to that between sexes in rats (Freedland et al., 1962; Brenneman and Kaufman, 1965; Dhondt et al., 1979).

The developmental pattern of PAH differs among species. In rats, for example, PAH activity is low during fetal development and develops rapidly after birth (Reem and Kretchmer, 1957; Freedland et al., 1962; Friedman and Kaufman, 1971; McGee et al., 1972; Tourian et al., 1972; Barranger, 1976; Dhondt et al., 1979; Yeoh et al., 1988). Substantial PAH activity appears to exist prior to birth in guinea pigs, humans, and monkeys (species unknown) (Friedman and Kaufman, 1971; Murthy and Berry, 1975). The pattern in chickens appears to be similar to that of guinea pigs and primates.

The present study was conducted in part to determine whether the chick embryo might serve as a model system with which to investigate the effects of Phe and its metabolites without the interference of maternal or fetal PAH activity. The present studies and those of Strittmatter and Oakley (1966), together with previous research involving the conversion of $^{14}$C-Phe to $^{14}$C-Tyr (Austic and Grau, 1971), suggest that the window of opportunity for such studies (i.e., a time when PAH activity is absent) is limited to a period from the beginning of incubation to an as yet undetermined time between 6 and 11 d of embryonic development.

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REFERENCES


