kaline phosphatase, indicating that they do not control capacity to synthesize the enzyme, but rather must play a role in determining rate of secretion into the serum, or in destroying or inhibiting the activity of the enzyme after it is secreted into the serum.

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Received July 15, 1966. P.S.E.B.M., 1966, v123.

The Phenylalanine-Hydroxylating System of Avian Liver. Pattern of Appearance During Embryonic Development and Limiting Component.* (31505)

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The inability of livers in phenylketonuric individuals to convert phenylalanine to tyrosine(1-3) has prompted attempts in several laboratories(4-7) to characterize the phenylalanine-hydroxylating system of mammalian liver. The overall process catalyzed by the system involves two separate reactions, which may be formulated as follows:

(1) Phenylalanine + tetrahydropteridine + O_2 → tyrosine + dihydropteridine + H_2O

(2) Dihydropteridine + NADPH‡ + H^+ → tetrahydropteridine + $NADP^+$

(3) Sum: Phenylalanine + O_2 + NADPH + H^+ → tyrosine + $NADP^+$ + H_2O

While the specific phenylalanine hydroxylase catalyzing Reaction 1 appears to be localized in liver, the enzyme system catalyzing Reaction 2, reduction of the pteridine cofactor, has been prepared from several mammalian tissues. The cofactor, which is closely related to dihydrobiopterin(8), may be replaced by certain synthetic pteridines in *in vitro* assay systems(7). It has been demonstrated that in the genetic disease phenylketonuria, the pteridine cofactor and the cofactor reductase are present(9) but the phenylalanine hydroxylase is missing(3,9,10).

The initial studies on development of the phenylalanine-hydroxylating system in rats indicated that the activity of the overall system is negligible in the livers of fetal rats or rats less than 24 hours old, and that the activity approaches the adult liver level several days after birth(11-13). It was further

* This work was supported by research grant GM-09304 from Nat. Inst. Health.

† Summer fellow, supported by a General Research Support Grant from Nat. Inst. Health to Bowman Gray School of Med.

‡ Abbrevations: NADP and NADPH, oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; DMTP, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

concluded that the missing component in the liver of newborn animals is phenylalanine hydroxylase(13). However, subsequent studies reported that the activity of the phenylalanine-hydroxylating system in livers of newborn rats is about 30%(14) and about 50 to 70%(15) of the adult levels in the absence of added cofactor. Further, from the effects of supplementation with cofactor and enzyme fractions, it was concluded that the limiting component in the newborn rat is not the phenylalanine hydroxylase but rather a lower level of pteridine cofactor and, possibly, of pteridine reductase(15).

The present study was undertaken to determine the nature and the pattern of appearance of the hepatic phenylalanine-hydroxylating system during the embryonic and post-hatching development of the chick, since the characteristics of avian development might impose patterns and controls of development for this system differing from those for the mammalian system, and since information regarding the avian system might contribute toward our understanding of the mammalian system. Attempts were also made to identify the limiting factor responsible for the low activity level of the avian system during early development.

Methods. Enzyme preparations. Fertile eggs of the Vantress-Leghorn strain were incubated at 38°C and 65% humidity in the laboratory. The stage of development of the embryos at the time of harvest was determined by the scale of Hamburger and Hamilton(16). The age of embryos is reported as the average incubation time given for each stage by Hamburger and Hamilton; these times agreed well with the actual periods of incubation. Chicks after hatching were provided with water and Purina starter chow *ad libitum*. Adult male rats of the Wistar strain were used for mammalian liver preparations.

Freshly excised livers were rinsed, blotted and weighed to obtain wet weight; embryo livers were pooled. The livers were homogenized in 3 volumes of 0.05 M phosphate buffer, pH 6.7, at moderate speed in a Potter-Elvehjem homogenizer fitted with a Teflon pestle to achieve essentially complete cell breakage, and the homogenates adjusted to contain 0.2 g liver per ml. Extracts of two types were obtained by centrifuging such homogenates either for 10 minutes at 15,000 $\times$ *g* (for E-1 extracts) or for 30 minutes at 100,000 $\times$ *g* (for E-2 extracts), then drawing off and making up the supernatant solution to the volume of homogenate used. Aliquots of extracts E-1 or E-2 were taken to 67% saturation with saturated, neutralized $(NH_4)_2SO_4$ solution, and the precipitates recovered by centrifugation for 10 minutes at 12,000 $\times$ *g*. Each precipitate was dissolved in 0.05 M phosphate buffer, pH 6.7, to give a volume 20% that of the volume of extract used; this represented the "Standard Precipitate" preparations (type SP-1 and SP-2, respectively). All operations were carried out at 0-4°C, and the preparations were assayed either immediately or after storage overnight at —15°C. With the standard chick embryo and chick preparations, this storage procedure entailed no significant loss of activity, but activity declined gradually on prolonged storage at —15°C, and rapidly on storage above freezing. Rat liver preparations were more labile, and were used immediately after preparation.

Crude separation of the phenylalanine-hydroxylating system into two fractions was achieved by fractionation of rat or chick liver E-1 extracts with saturated $(NH_4)_2SO_4$, essentially by the procedure of Mitoma(5). In preparations from rat liver, Fraction 1, precipitated between 25% and 40% saturation, contains largely the phenylalanine hydroxylase activity, while the Fraction 2, precipitated between 45% and 67% saturation, contains largely the cofactor reductase activity(6,7). Each precipitate was dissolved in 0.05 M phosphate buffer, pH 6.7, to 20% of the original volume of E-1 used. Since Fraction 1 was very unstable, assays involving these fractions were performed immediately after preparation.

Phenylalanine hydroxylation. An adaptation of the method of Mitoma(5) was used to assay phenylalanine-hydroxylating activity. Enzyme fraction in a total volume of 2 ml, containing 0.05 M phosphate buffer, pH 6.7, 0.001 M NADH,‡ 0.003 M nicotinamide, and 0.006 M phenylalanine, was incubated with

shaking for 30 minutes at 37°C. The reaction was stopped by addition of 10% trichloroacetic acid; in controls, trichloroacetic acid was added before enzyme. The flask contents were centrifuged, and aliquots of the supernatant solutions assayed for tyrosine by the colorimetric method of Udenfriend and Cooper (17). Unless otherwise noted, results are expressed as μmoles tyrosine formed per hour by the amount of enzyme preparation obtained from 1 g liver. Reduced pyridine nucleotide was essential for the reaction; NADPH could substitute for NADH with essentially the same rate of reaction. Increasing the reduced pyridine nucleotide concentration above the level specified or substituting an NADPH-generating system did not substantially increase the reaction rate. Controls showed that tyrosine was not metabolized at a significant rate under the standard assay conditions by the enzyme preparations used.

Protein assays. Protein was determined by the micromethod of Lowry *et al*(18) with crystalline bovine serum albumin as standard.

Materials. NADH and NADPH were obtained from Sigma Chemical Co.; 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (DMTP‡) from California Corp. for Biochemical Research; L-phenylalanine, L-tyrosine and tetrahydrofolic acid from Nutritional Biochemicals Corp. Other chemicals used were of reagent grade.

Results. Development of phenylalanine-hydroxylating system. Fig. 1 shows the pattern of appearance of overall phenylalanine-hydroxylating capacity in the livers of developing chick embryos and chicks, as determined by assays of homogenates and, more extensively, of SP-2 preparations. The SP-2 preparations, which were chosen for assay to minimize possible effects of free, low molecular weight components, showed activities only 15-25% of the corresponding homogenates, but the developmental pattern was very similar for homogenates and SP-2 preparations. The activity rose from a low level in the 12-day embryo liver to a peak at 18 days, then decreased slightly to a plateau after hatching, with an apparent minimum 2 to 3 days after hatching. Also shown in Fig. 1 are results obtained with liver preparations of types similar to those used in previous studies on the mammalian system, including E-1 and E-2 extracts and SP-1 preparations. These 3 types of preparations showed the same developmental pattern found with the homogenates and SP-2 preparations, with levels about 80, 70 and 40%, respectively, of the homogenate activities at corresponding stages of development.

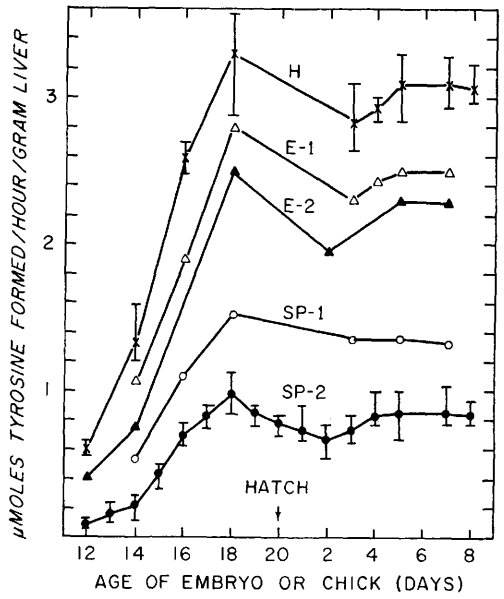

FIG. 1. Development of phenylalanine-hydroxylating system in avian liver. Different types of preparations from liver of chick embryos and chicks of various developmental ages were assayed for capacity to form tyrosine from phenylalanine, as described under *Methods*. Curve H, homogenate; Curve E-1, extract after centrifugation of homogenate at 15,000 × *g*; Curve E-2, extract after centrifugation of homogenate at 100,000 × *g*; Curve SP-1, $(NH_4)_2SO_4$-precipitated preparation from E-1 extract; Curve SP-2, $(NH_4)_2SO_4$-precipitated preparation from E-2 extract. In curves H and SP-2, each point shows the mean and range of values from 2 to 7 preparations; in curves E-1, E-2 and SP-1, each point is the mean of values from 1 to 5 preparations.

Components of enzyme system. To test whether the avian liver system involves two separate enzyme components, like the mammalian phenylalanine-hydroxylating system, E-1 extracts from 5-day chick liver were fractionated by $(NH_4)_2SO_4$ precipitation, as described under *Methods*, into Fraction 1 and Fraction 2, which in analogous preparations from rat liver contain primarily the phenyl-

TABLE I. Phenylalanine Hydroxylase Activity in Chick Liver Fractions and with Combinations of Chick and Rat Liver Fractions.

Liver preparations	Activity*
Chick E-1 extract	.260
" fraction 1	.007
" " 2	.007
" " 1 + chick fraction 2	.067
" SP-1 preparation	.140
" fraction 1 + rat fraction 1	.030
" " 1 + " " 2	.065
" " 2 + " " 1	.060
" " 2 + " " 2	.026
Rat fraction 1	.020
" " 2	.015
" " 1 + rat fraction 2	.190

* μmoles tyrosine formed in 30 min with preparations equivalent to following weights of original liver: each preparation from 5-day chick, 0.2 g; each rat fraction, 0.1 g.

alanine hydroxylase activity and the cofactor reductase activity, respectively. Assays of the chick liver fractions singly and in combination with each other and with rat liver fractions are summarized in Table I. Chick Fractions 1 and 2 singly showed very minor activity, while their combination restored a significant portion of the original E-1 extract activity; the restoration of activity, although incomplete, represented a large portion of the total activity that could be recovered by $(NH_4)_2SO_4$ precipitation, as exemplified by the activity of SP-1. With combinations of chick and rat liver fractions, significant stimulation above summation of activities was obtained by combination of complementary fractions but not of analogous fractions. The results suggest that the partially separated activities of chick Fractions 1 and 2 represent, respectively, phenylalanine hydroxylase and cofactor reductase, analogous to the components of the mammalian liver system.

To test the possible involvement of free, dissociable or labile cofactor in the avian system, SP-1 and SP-2 preparations from 14-day and 18-day chick embryo livers were supplemented with tetrahydrofolate, with the reduced synthetic cofactor, DMTP[‡], or with crude "cofactor extracts" obtained from chick liver or rat liver by brief heating of liver homogenates, followed by centrifugation, as described by Brenneman and Kaufman(15). With SP preparations from 0.5 g liver of either 14-day or 18-day embryo, supplementation with "cofactor extract" from 0.5 g chick or rat liver produced only minor and variable stimulation (0 to 50% increase), while 0.005 M tetrahydrofolate or 0.0005 M DMTP produced 3 to 5 fold stimulation.

Limiting component. In attempts to identify the limiting component whose deficiency is responsible for the relatively low capacity of the phenylalanine-hydroxylating system in early chick embryo liver, preparations from livers of various developmental stages were supplemented singly and in combination with DMTP, rat Fraction 1 and rat Fraction 2. Typical results with liver homogenate and SP-1 preparations are shown in Table II; results with E-1 preparations (not shown) were similar to the pattern seen with homogenates. All SP-1 preparations were markedly stimulated by DMTP, but the level in supplemented early embryo (14-day) preparation was not as high as in supplemented late embryo or chick preparations. In contrast, no homogenates were stimulated by DMTP. Rat Fraction 1 (containing phenylalanine hydroxylase) stimulated SP-1 preparations from each developmental stage, and also caused a marked stimulation of early embryo homogenate; the supplementation raised the activity of both early embryo preparations to essentially the same level as seen in the comparable supplemented preparations from later developmental stages. In contrast, rat Fraction 2 caused no marked stimulation in any preparation. The apparent marked stimulation of SP-1 preparations by the combination of DMTP plus rat Fraction 1 and the lesser stimulation by the combination of DMTP plus rat Fraction 2 apparently reflect primarily a stimulation of the rat fractions by DMTP, as indicated by the control experiments in Table II.

The low activity of early embryo preparations did not appear to result from the presence of an inhibitor, because when homogenates of liver from 18-day embryos or chicks were assayed in the presence of equivalent amounts of 12-day or 14-day embryo liver homogenates, a summation of the separate activities was observed in each case (data not shown).

TABLE II. Stimulation of Avian Liver Preparations by Pteridine Cofactor and by Rat Liver Enzyme Fractions.

Chick liver preparation	Phenylalanine hydroxylase activity* with supplement noted: None	DMTP†	Rat fraction 1	Rat fraction 2	DMTP + rat fraction 1	DMTP + rat fraction 2
14-day embryo SP-1	.03	.18	.60	.06	2.33	.66
18- " " " "	.18	.48	.70	.26	1.90	1.10
5- " chick " "	.17	.42	.56	.17	2.22	.86
14- " embryo homogenate	.12	.11	.45	.12	.93	.76
18- " " "	.33	.34	.52	.43	1.05	1.08
5- " chick "	.32	.29	.37	.42	1.08	1.08

Control activities: Rat fraction 1 = .09; rat fraction 2 = .03; rat fraction 1 + 2 = .89; rat fraction 1 + DMTP = 2.21; rat fraction 2 + DMTP = .86.

* μmoles tyrosine formed in 30 min with preparations equivalent to following weights of original liver: SP-1 preparations, 0.25 g; homogenates, 0.2 g; rat fractions, each 0.5 g.

† 0.0005 M DMTP.

Discussion. The present study suggests that the avian liver phenylalanine-hydroxylating system is similar to the mammalian system in its components and characteristics. Two enzyme fractions, replaceable by crude preparations of mammalian phenylalanine hydroxylase and pteridine cofactor reductase, respectively, are involved, and the stimulation by tetrahydrofolate and DMTP suggests involvement of a pteridine cofactor as in the mammalian system, although the preliminary studies with crude cofactor extracts do not clearly establish the presence of a natural pteridine cofactor. Incomplete recovery of activity in precipitated preparations may reflect partial dissociation of cofactor or a high lability of some component in the system. The major portion of activity was recovered in the supernatant solution following centrifugation of homogenates in electrolyte medium at moderate or high centrifugal force (Curves E-1 and E-2 in Fig. 1), in agreement with reports that much or all of the mammalian system is recovered in similar extracts (5,11-15). However, the consistently lower recovery from preparations subjected to higher centrifugal forces (Curves E-2 and SP-2 in Fig. 1) suggests some particulate association, either intrinsic or by procedural artifact, of some component or portion of the total activity.

The developmental pattern of the avian system shows a definite rise from a low level in the early embryo to a peak activity shortly before hatching. The apparent slight decline of activity at the time of hatching, when activities are expressed on a liver wet weight basis (Fig. 1), is of uncertain significance, particularly as there is at this time a marked increase in the dry weight:wet weight ratio of liver and an accumulation of lipids, without a similar marked increase in soluble protein (19). Attainment of peak activity prior to hatching in the avian system is in apparent contrast to the mammalian system, where maximal activity is reportedly not reached until some time after birth (11-15), although a recent study(15) indicates that a major percentage of the adult level is already present in newborn rats.

The data of Table II suggest that the primary limiting factor responsible for the relatively low level of phenylalanine-hydroxylating capacity in the early chick embryo is a relative lack of active phenylalanine-hydroxylase component. Further studies will be required to determine whether this apparent deficiency of active hydroxylase reflects a lower rate of enzyme protein formation, a relative lack of some cofactor to complete the active enzyme, or a greater turnover or lability of the early embryo enzyme. SP preparations of all developmental stages were markedly stimulated by DMTP, but since the activity of early embryo SP was not raised to the level of late embryo or chick SP, the stimulation does not suggest a lack of enzyme-bound cofactor as the factor responsible for the lower activity in early embryo liver. The stimulation of all SP preparations by DMTP might reflect a similar cofactor deficiency at all stages or a similar cofactor loss or destruction

in each preparation; alternately, it may represent an artificial stimulation above the level of activity with cofactor-saturated enzyme, with the DMTP functioning as a free intermediate carrier facilitating transport between components of the enzyme system. Failure of DMTP to stimulate homogenate activities probably reflects rapid inactivation of the labile reduced cofactor by oxidative systems of the homogenates, rather than presence of maximal levels of natural cofactor, since stimulation of the rat fractions by DMTP was not observed in the presence of homogenates.

The limiting component in development of the avian system may therefore be different from that of the mammalian system, in which, according to the most recent study(15), the primary deficiency is the pteridine cofactor. Rat liver preparations of all ages were markedly stimulated by DMTP, but the relatively low activity of preparations from livers of newborn rats was raised by DMTP to the same levels as found with DMTP-supplemented preparations from adult rat liver.

Summary. The phenylalanine-hydroxylating system of chick liver was found to resemble the mammalian liver system in characteristics and components, with apparent involvement of a phenylalanine hydroxylase, a pteridine cofactor and a cofactor reductase. Studies on the pattern of appearance of the avian liver system during embryonic and post-hatching development of the chick showed that the activity rises from a low level in early embryo to a peak shortly before hatching, in apparent contrast to the developmental pattern reported for the mammalian system. Also in apparent contrast to recent studies on the mammalian system, the limiting factor primarily responsible for the pattern of development in avian liver appeared to be a relative lack of active phenylalanine hydroxylase component in the early chick embryo.

The authors wish to acknowledge the technical assistance of Mr. Ernest Jones and Mr. Walter Wiley.

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Received July 18, 1966. P.S.E.B.M., 1966, v123.