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THE FORMATION OF HISTAMINE BY FETAL RAT LIVER*

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Abstract—Some of the characteristics of the histidine decarboxylase derived from fetal rat liver have been determined. The pH optimum is 6·6; pyridoxal 5′-phosphate is required as a coenzyme; the enzyme is not appreciably inhibited by α-methyl DOPA; and benzene neither activates nor inhibits the enzyme. The fetal enzyme activity appears shortly after the appearance of liver cells, reaches a peak at the fourteenth day of gestation and apparently disappears rapidly after birth. At the present time the functional significance of this enzyme remains obscure.

THE observation that the rat fetus can synthesize relatively large quantities of histamine was first made by Kahlson and co-workers.[1] Using radioactive histidine, these authors demonstrated histidine decarboxylase activity in fetal liver, but did not describe the characteristics of the enzyme. Recently Ganrot *et al.*[2] and Telford and West[3] have described some of the characteristics of the fetal liver enzyme. The present paper presents additional data concerning this enzyme.

METHODS

Characterization of enzyme

Livers from approximately 18-day-old fetuses (Long–Evans strain) were homogenized in either 9 or 19 parts of cold isotonic KCl. The homogenates were spun, at 100,000 × *g*, for 1 hr in a Spinco Model L ultracentrifuge; the supernatant fraction was used for the enzyme assay.

For determination of enzyme activity the supernatant fraction, KH_2PO_4 (0·1 M, pH 6·6), 50 μg of pyridoxal 5′-phosphate (PP), 5·5 mg of L-histidine·HCl·H_2O, and isotonic KCl in a final volume of 5·4 ml were incubated at 37 °C under N_2 in a Dubnoff metabolic shaker. At the end of the incubation time, 0·6 ml of 6 N $HClO_4$ was added to stop the reaction and precipitate the proteins. The $HClO_4$-supernatant fraction was then analysed for histamine formed during the incubation. Controls consisted of the complete system with the $HClO_4$ added before the incubation.

"Dialysis" of the enzyme was accomplished by passing portions of the liver supernatant fraction through a Cephadex (G-25) column in the cold. Carrying out this treatment in the cold prevented loss of apoenzyme, as shown by the fact that full enzyme activity was restored by addition of PP.

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Enzyme activity during gestation

Long–Evans rats were mated and vaginal smears taken to determine presence of sperm. The first day in which sperm were seen was designated day-zero. Starting at day-12, pregnant females were stunned and decapitated. The fetuses were removed and placed on a cooled tray. The livers were removed, pooled and a 5 per cent homogenate in cold isotonic KCl was prepared. The enzyme activity of 1-ml aliquots of the liver homogenate was determined by using a procedure similar to that described for the liver supernatant fraction. At zero-time and at 30 min, 1-ml aliquots of the incubation mixture were added to tubes containing 1 ml of 1·2 N $HClO_4$, and then analysed for histamine.

Small samples (10 μl) of the 5 per cent homogenate were analysed for protein, using a slight modification of the procedure of Lowry.[4] The modification consisted of changing the volumes and limiting the amount of protein to 30 to 90 μg per sample.

Histamine assay

The $HClO_4$-extracts were analysed for histamine content by using essentially the fluorimetric procedure of Shore *et al.*[5] A few changes, as described below, were made in the original method, the most important being a change in the extracting solvent; this was necessary because of the presence of large amounts of histidine in the incubation mixture. It was found that a 3:2 mixture of *n*-butanol and chloroform extracted only about 0·3 per cent of the histidine, while *n*-butanol alone, as used in the original method, extracted about 3 per cent. This solvent mixture extracted approximately 90 per cent of the histamine. Thus, by using the solvent mixture, and then washing the solvent with an alkaline NaCl solution, 81 per cent of the histamine was extracted, while the yield of histidine in the final extract was negligible. It should be pointed out that impurities, especially aldehydes, in the solvents can markedly reduce the recovery of histamine. Reagent grade *n*-butanol from either the Fisher Chemical Co. or Eastman Kodak Co. has been used in the author's laboratory.

It is important to note that the reaction time for condensing histamine with the *o*-phthalaldehyde is somewhat dependent upon the ambient temperature of the laboratory. Higher temperatures (greater than 68 °F) shorten the time for optimal fluorophore formation and also make the optimal time critical; at 68 °F not much difference is observed between reaction times of $3\frac{1}{2}$ and $4\frac{1}{2}$ min.

In the experiments reported in this paper the following procedure was used: to 2-ml aliquots of 0·6 N $HClO_4$-extracts in a 15-ml centrifuge tube was added 0·3 ml of 5 N NaOH and 1 g of NaCl, followed by 10 ml of *n*-butanol–chloroform solvent mixture. The mixture was shaken in a mechanical shaker for 5 min, centrifuged to separate the layers, and the organic phase was transferred to another centrifuge tube containing 2 ml of NaCl-saturated 0·1 N NaOH. This was shaken for 1 min, centrifuged, and 8 ml of the organic solvent were transferred to a 40-ml centrifuge tube containing 5 ml of 0·1 N HCl and 15 ml of *n*-heptane. This mixture was shaken for 1 min, centrifuged, and then 2-ml aliquots of the 0·1 N HCl-layer were analysed for histamine, as described by Shore *et al.*[5] The fluorescence was measured in an Aminco–Kiers Spectrophosphorimeter adapted for spectrophotofluorometry. Internal standards were determined and the reliability of the method was ascertained by adding 1 μg of histamine base to a $HClO_4$-extract. Recoveries of added histamine were consistently around 85 per cent.

RESULTS

Characteristics of the enzyme

Fig. 1 illustrates the effects of varying pH and substrate concentration on enzyme activity. The pH optimum is approximately 6·6, a finding which confirms the observations of Telford and West.[3] The maximal rate of reaction was obtained at a substrate concentration between 10^{-2} and 10^{-3} M. In all subsequent experiments the pH was adjusted to 6·6 and a substrate concentration of $4{\cdot}76 \times 10^{-3}$ M (1 mg of L-histidine·$HCl{\cdot}H_2O$ per ml) was used.

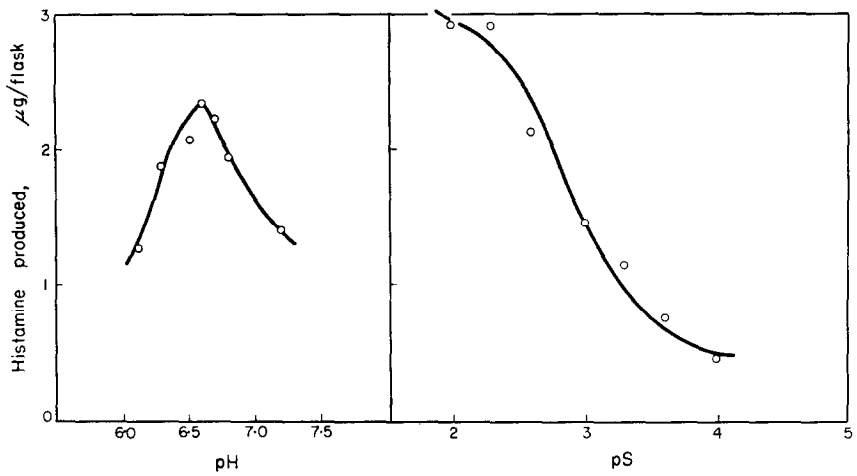

FIG. 1. The influence of pH and substrate concentration on the activity of the histidine decarboxylase of fetal rat liver. The conditions of the experiments are given in text.

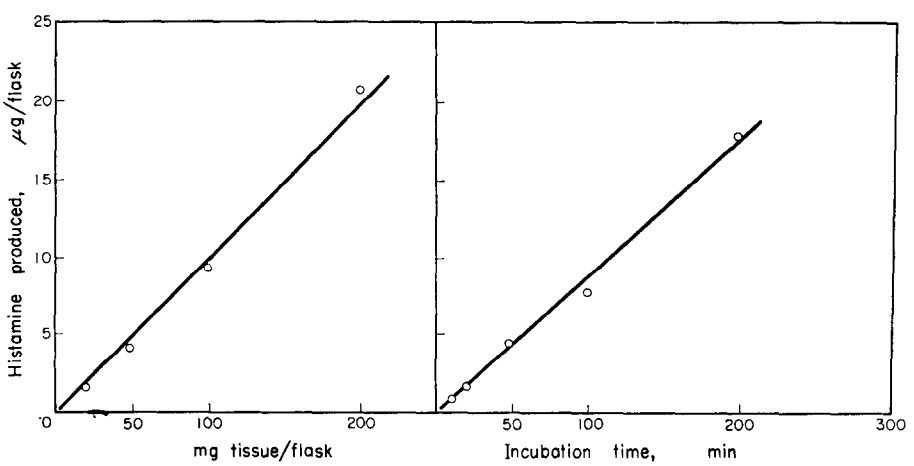

FIG. 2. The influence of tissue concentration and incubation time on the rate of activity of the histidine decarboxylase of fetal rat liver.

Fig. 2 illustrates the effects of tissue concentration and time of incubation. As can be seen, the enzyme activity varies linearly with both of these variables within the limits used. The amount of histamine formed by 20 mg of tissue in 30 min was sufficient for a reliable assay; accordingly, these conditions were used in later experiments.

Minimum amounts of tissue decrease the probability of non-specific binding of coenzyme, substrate, and product; and the short incubation time gives the initial rates of reaction and decreases the probability of bacterial contamination.

The requirement for PP is shown in Fig. 3. In these experiments the supernatant fraction of the fetal liver was passed through a column of Cephadex (G-25) in the cold before assaying for enzyme activity. This procedure removed the small ions, a technique equivalent to dialysis of the preparation. As seen from the figure, the treated supernatant fraction in the absence of added PP, had less than 10 per cent of the maximal activity; in similar experiments, this treatment caused a loss of all activity. Addition of very small amounts of PP restored full activity. In all other experiments, excess amounts of PP were added.

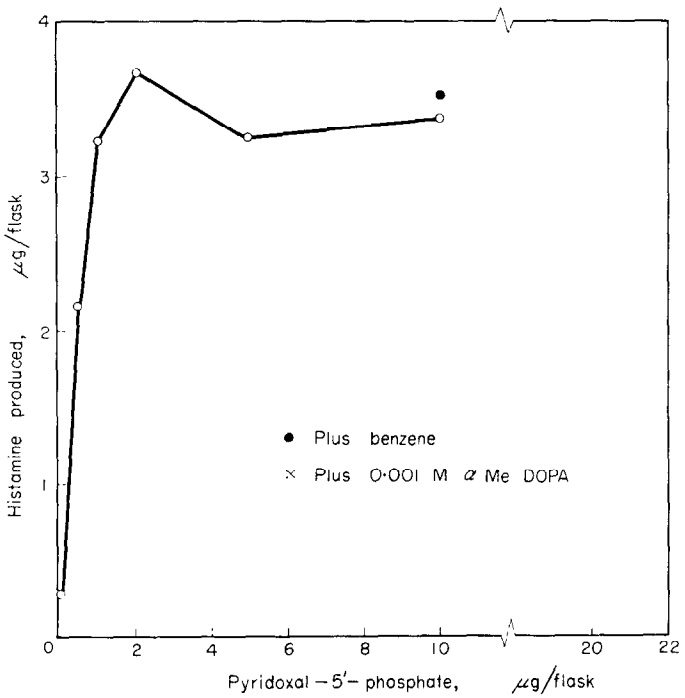

FIG. 3. The effect of adding pyridoxal 5'-phosphate on the activity of an homogenate of fetal rat liver treated with Cephadex (G–25) to remove the small ions. The effects of adding benzene or α-methyl DOPA also are shown.

Also shown in Fig. 3 are the results of adding benzene and α-methyl-3:4-dihydroxyphenylalanine (α-methyl DOPA) to the incubation mixtures. Benzene has been reported to activate certain mammalian histidine decarboxylases,[6] but adding a small amount (1 drop) to an incubation mixture having excess PP did not influence the fetal liver enzyme. α-Methyl DOPA is a potent inhibitor of some other mammalian decarboxylases,[7] but at a concentration of 10^{-3} M it produced an inhibition of the fetal enzyme of only 31 per cent. This inhibition was overcome by adding more PP, a finding which indicated that the inhibition was probably attributable to some interference with the PP, rather than to competition with substrate. Other experiments supported this view, for it was noted that the greatest inhibition took place if the

α-methyl DOPA and PP were mixed prior to incubation. Inhibition was also greater if the α-methyl DOPA was added before the PP. Almost no inhibition occurred at concentrations of α-methyl DOPA of 0·01 M, if the concentration of PP was 25 μg per ml. The substrate specificity of the enzyme was not determined; however, no decarboxylation of 5-hydroxytryptophan by fetal liver was demonstrated when conditions were optimal for histidine decarboxylation.

Using conditions optimal for the liver enzyme, the following tissues of 21-day fetuses were assayed: heart, lung, pancreas, spleen, brain, kidney, adrenal, skin, small intestine, stomach, and skeletal muscle. The pancreas had 5 per cent of the activity of the liver; all other tissues had less than 1 per cent.

The liver enzyme was heat-stable at 50 °C for 3 min, but was destroyed rapidly at 100 °C. Very little activity was lost when the supernatant fractions were frozen and kept in a deep freeze for 6 weeks.

Enzyme activity during gestation

Kahlson *et al.*[8] have reported that pregnant rats, starting about the fifteenth day of pregnancy, excrete large amounts of free histamine in the urine. Histamine excretion increased until birth occurred and then rapidly returned to normal. It was of interest to know whether the increasing amounts were merely a reflection of the increasing size of the liver, with a concomitant increase in enzyme content, or whether the enzyme concentration of the fetal liver varied during gestation.

The methods used in assaying enzyme activity have been described; however, it should be pointed out that optimal conditions were used, with excesses of substrate and coenzyme, so that the limiting factor would be the enzyme concentration. To minimize variations attributable to different water-content of fetal tissue during gestation, enzyme activities were based on the amount of protein present.

The results are shown graphically in Fig. 4. Basing results on protein-content decreased the spread of values obtained when these were compared with results based upon tissue-weight. The enzyme activity, and presumably concentration, increased rapidly in the 2 days following day-12. A peak at day-14 was noted; then the activity dropped to a fairly constant value until birth occurred. Thereafter, the activity dropped markedly and it is doubtful that any fetal enzyme remained. These results are similar to those of Telford and West,[3] who used different methods for determining enzyme activity. The essential difference in the two studies is the addition of PP in the author's system, in order to produce optimal conditions for enzyme activity.

DISCUSSION

Various properties of an active histidine decarboxylase found in fetal rat liver have been presented. While most of the observations agree with and confirm the results of others,[2, 3] there are two apparent disagreements. First, Ganrot *et al.*[2] reported that the K_m of the fetal liver enzyme was 2×10^{-5} M. Although the experiments presented in this paper were not designed to determine the K_m, an estimate of 10^{-3} M can be made from the data of Fig. 1. A possible reason for the apparent difference in these values is that the assay conditions used by Ganrot *et al.*[2] were not optimal. For example, Ganrot *et al.*[2] incubated at pH 7·8, the author at pH 6·6. Second, Telford and West[3] reported that addition of pyridoxal to their incubation mixture did not cause an increase in enzyme activity. This implies that pyridoxal or a similar compound

(PP) is not a necessary cofactor. Such an implication is misleading, since the present work shows clearly that the treatment with Cephadex removed a necessary cofactor and that full activity was restored by adding minute amounts of PP to the treated preparation. While this does not prove that PP was the factor removed, it strongly suggests this possibility. Since Telford and West[3] did not add PP to their incubation mixtures, and their results with respect to the enzyme activity during gestation agree with those of the author, it can be concluded that during gestation the fetal liver normally contains enough cofactor (PP) and that a non-dialysed preparation does not require the addition of exogenous PP. It should be emphasized that enzyme activity *in vitro* depends upon the concentration not only of the apoenzyme, but also of the required cofactors; therefore, to insure maximal *in vitro* activity, addition of cofactors would be required.

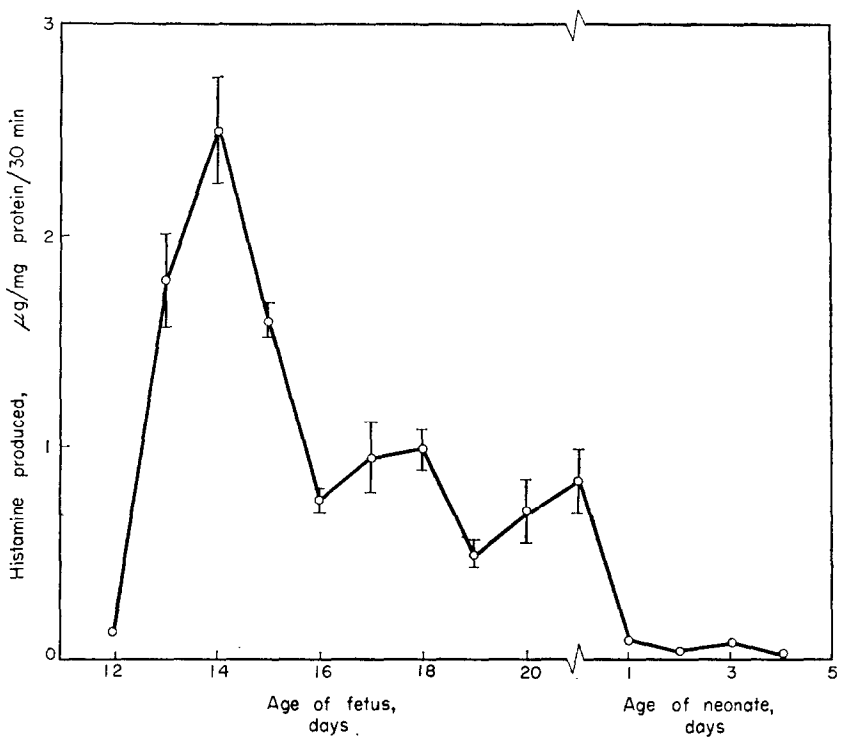

FIG. 4. The histidine decarboxylase of fetal rat liver during gestation. The values are means ± the standard deviations of from 3 to 8 determinations. The plain circles represent the average of 2 determinations.

In the light of the present results, together with those of others,[2, 3, 6, 7, 10–14] it is clear that the enzyme in fetal liver is not identical with the histidine decarboxylases found in many other tissues. Some of the characteristics of these enzymes are shown in Table 1. It is clear from the data in Table 1 that the characteristics of the fetal enzyme are similar to those of the enzyme found in mouse mast cell tumor tissue and are different from those of enzymes from other tissues. The histidine decarboxylase activity of kidney, brain and adult liver seems to be attributable to the activity of a

more general α-amino acid decarboxylase that also decarboxylates 5-hydroxytryptophan and 3:4-dihydroxyphenylalanine,[2, 15] whereas the enzyme in fetal rat liver, like the enzyme found in mouse mast cell tumors, appears to be more specific for histidine.

The activity, and presumably concentration, of the enzyme in the fetal liver is not constant throughout gestation. The appearance of this enzyme activity about the twelfth day of gestation, the peak activity at the fourteenth day of gestation, and the very rapid disappearance of the acitivity following birth are noteworthy points to

TABLE 1. CHARACTERISTICS OF THE HISTIDINE DECARBOXYLASE ACTIVITY FOUND IN VARIOUS TISSUES

	Value or effect	Source of enzyme	Reference
pH optimum	6·6	Fetal rat liver	This paper
	6·5	Fetal rat liver	3
	6·0	Mouse mast cell tumor	11
	7·0	Mouse mast cell tumor	10
	7·2	Rat skin, stomach, brain	12
	7·2–7·4	Rabbit platelet	14
	7·4	Rat peritoneal mast cells	13
	8·0	Rabbit kidney cortex	12
	8·0	Adult rat liver	3
	9·0–9·5	Guinea pig kidney	11
K_m	10^{-3} M*	Fetal rat liver	This paper
	2×10^{-5} M	Fetal rat liver	2
	5×10^{-4} M	Mouse mast cell tumour	11
	10^{-1} M	Rabbit kidney cortex	2
	5×10^{-2} M	Guinea pig kidney	11
Inhibition by α-methyl DOPA	None at 10^{-3} M*	Fetal rat liver	This paper
	Very weak	Fetal rat liver	2
	None at 10^{-3} M	Mouse mast cell tumour	11
	100% at 10^{-3} M	Guinea pig kidney	11
	50% at 7×10^{-7} M	Guinea pig kidney	7
Effect of benzene	None	Fetal rat liver	This paper
	None	Fetal rat liver	3
	None	Mouse mast cell tumor	11
	7-fold stimulation	Rabbit kidney	6, 12
	40-fold stimulation	Adult rat liver	3
	80% inhibition	Rat stomach	12
	2–3-fold stimulation	Guinea pig kidney	11

* See text.

consider in delineating the physiologic significance of this fetal enzyme. Appearance of the activity follows the development of liver cells in the fetus. Since no other tissue had any significant amount of histidine decarboxylase activity, the latter appears definitely to be associated with hepatic tissue. The great change in enzyme activity at about day-13 occurs at a time when there is a large change in fetal circulation that involves the liver,[9] and it is possible that at this time factors brought to the liver from the maternal bloodstream influence the enzyme activity. Conversely, the change in enzyme activity, with the resultant increased production of histamine, might also be important in causing or aiding such vascular changes.

The rapid decline of activity following birth indicates that the enzyme activity may be dependent upon the fetal state. Supporting this suggestion are the following

observations: first, there was a rapid decline in enzyme activity in animals removed prematurely from the mother; and second, a sustained enzyme activity occurred in fetuses whose birth was delayed by injecting progesterone into the female. The possibility that the fetal enzyme is associated with some fetal function of the liver, such as hematopoiesis, has been considered, but as yet no positive results have been obtained. In addition, attempts to influence enzyme activity by injecting substrate into either the pregnant female or the newborn have not been successful.

It must be concluded that neither the function of the histidine decarboxylase in the fetal rat liver nor the role of the large quantities of histamine formed by this enzyme is clear. However, future study designed to investigate these questions should be facilitated by knowledge of some of the properties of this fetal enzyme.

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