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HISTIDINE DECARBOXYLASE IN THE FETAL RAT

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Abstract—The properties of a purified preparation of histidine decarboxylase from fetal rat tissues have been studied. From kinetic data there is reason to believe that the enzyme requires the anionic form of the substrate and that the active form of the enzyme occurs more abundantly at acid pH. Pyridoxal-5-phosphate is required as a coenzyme. The purified enzyme seems fairly specific. No DOPA decarboxylase activity could be demonstrated. The results suggest that the histidine decarboxylase of fetal tissues is different from the enzyme present in the adult rabbit kidney.

KAHLSON *et al.* were first to report that tissues of the fetal rat—notably the liver—contain high histidine decarboxylase activity.[1] This observation was followed by similar findings in other tissues characterized by rapid growth.[2–5] The properties of the fetal enzyme have been studied by several investigators. Ganrot, Rosengren and Rosengren (1961) concluded from their results that the fetal histidine decarboxylase was entirely different from the histamine forming enzyme present in rabbit kidney,[6] the latter enzyme being apparently identical with L-3, 4-dihydroxyphenylalanine (DOPA) decarboxylase. Fetal histidine decarboxylase was not inhibited by DOPA decarboxylase inhibitors and was considered more specific. Other investigators, working with crude enzyme preparations, have given additional data on the fetal enzyme.[7,8] This paper reports the properties of a purified preparation of fetal histidine decarboxylase.

MATERIALS AND METHODS

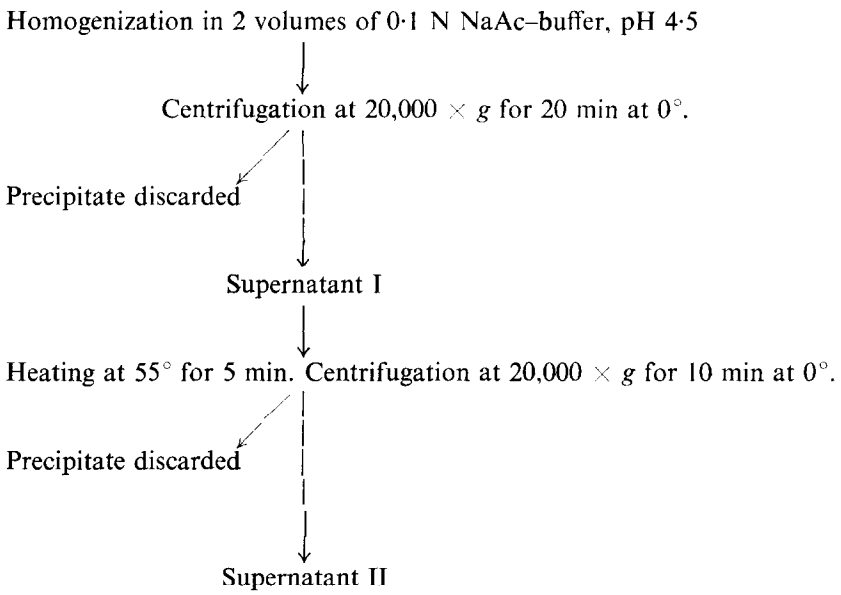
Radioactive chemicals: DL-3, 4, -dihydroxyphenyl-alanine-2-^{14}C (DOPA) with a specific activity of 5·75 mc/mM was furnished by the Radiochemical Centre, England. Before use the ^{14}C -DOPA preparation was purified on a Dowex 50 column according to a method that has been described elsewhere.[9]

L-Histidine (base) was delivered by Hoffmann-La Roche. Pyridoxal-5-phosphate (92% purity) was delivered by L. Light & Co. Ltd. *o*-Phtalaldehyde was obtained from Fluka. All organic solvents were furnished by Merck.

Preparation of enzyme

Pregnant rats were decapitated 15–20 days after mating and the litters were removed and pooled. In frozen condition the fetal rat tissue maintained its enzyme activity for several weeks. At least 50 g whole fetus were taken for each preparation.

In order to increase the specific activity of the extract the following procedure for fractionation was used.



More than 90 per cent of the initial enzyme activity was present in supernatant II.

Fractionation of Supernatant II with $(NH_4)_2SO_4$

The enzymic material was precipitated with $(NH_4)_2SO_4$ at 25, 40 and 60 per cent saturation respectively. The precipitates were spun down, re-dissolved in 10–20 ml 0·1 M phosphate buffer, pH 7·0 and dialysed against re-distilled water at 4° over-night. Sediments developed on dialysis and the extracts were centrifuged before the assay of activity. The fraction which had precipitated at between 25 and 40 per cent saturation was found to contain most of the enzyme activity. This fraction was again treated with $(NH_4)_2SO_4$ and the material which now precipitated between 28 and 42 per cent saturation contained all enzyme activity. This extract was dialysed against water for at least 6 hr. This treatment resulted in a 200-fold purification of the enzyme as compared to Supernatant II. More than 50 per cent of the enzyme activity of the initial homogenate was preserved in this extract, which was diluted to contain 0·4 per cent protein before use. In this state the enzyme was found to be stable for several days. All studies on fetal histidine decarboxylase were made on preparations of this kind.

Protein determination. The Biuret method was used throughout. Human serium albumin (Kabi) was used as standard.

Assay of histidine decarboxylase activity

If not otherwise stated 0·1 ml of the enzyme preparation was incubated with various amounts of L-histidine in the presence of pyridoxal-5-phosphate in 1 ml 0·1 M phosphate buffer for 1 hr at 37° in nitrogen atmosphere. When high concentrations of histidine were used, pH had to be adjusted by the addition of small amounts of

0·01 N HCl. The enzyme was allowed to react with the coenzyme for 15 min before the addition of substrate. As a rule, 10 μg of coenzyme was added. When experiments using low histidine concentrations were performed the amount of coenzyme added was diminished in order to avoid Schiff base formation of substrate and coenzyme in excess. The molar concentration of coenzyme never exceeded that of the substrate. Blanks were obtained by reproducing the experimental conditions without the enzyme. To the standards varying amounts of histamine dihydrochloride were added as well.

Following incubation trichloroacetic acid was added to give a final concentration of 5% and a total volume of 3 ml. The resulting precipitate was spun down and discarded. The supernatants were washed twice with 4 volumes of ethyl ether to remove the trichloroacetic acid. The further procedure was in principle that described by Burkhalter.[8] The samples were transferred to centrifuge tubes containing 1 g NaCl and 0·6 ml 5 N NaOH and extracted for 5 min with 15 ml of a 3:2 mixture of n-butanol and chloroform. The organic phase was then transferred to another tube containing 4 ml salt-saturated 0·1 N NaOH. After shaking for 1 min the organic phase was transferred to another tube containing 3 ml 0·1 N HCl and 4 ml n-heptane. This mixture was shaken for 1 min and then centrifuged. The organic phase was sucked off. The acid extract was diluted 3 times with water and the final fluorimetric readings were made as described by Shore *et al.*[10] in an Aminco–Bowman spectrophotofluorometer. In a few experiments with low amounts of substrate (less than 1 μg histidine/ml), the histamine formed was isolated on a Dowex 50 column. In these experiments the preliminary procedure was exactly as described above. However, no butanol extraction was performed. Instead the pH of the sample was adjusted to about 8·0 by means of small amounts of 0·1 N NaOH and the sample was then transferred to a 100 mg Dowex 50 Na^+ column, previously treated with 10 ml 0·8 M phosphate buffer, pH 8·0 and 5 ml redistilled water. After passage of the sample the column was washed with 20 ml 0·03 M phosphate buffer, pH 8·0 and 5 ml redistilled water. All histidine passed through in the effluent while histamine had to be eluted with 6 ml N HCl. This fraction was evaporated to dryness on a steam bath and re-dissolved in 3 ml 0·01 N HCl. The measurements were made on this extract according to the method described above.

Enzyme specificity

The ability of the enzyme preparation to decarboxylate L-3, 4-dihydroxyphenylalanine (L-DOPA), was tested in a series of experiments with ^{14}C-labelled substrate. The enzyme was incubated with 3 μg ^{14}C-DOPA in the presence of pyridoxal-5-phosphate under conditions as described for the experiments on histidine decarboxylase. Blank values were obtained by identical incubations without enzyme. The incubation was interrupted by the addition of 4 ml 0·4 N perchloric acid containing 10 μg dopamine. The precipitate was spun down and the supernatant was neutralized with 2 N potassium carbonate to a final pH of about 6·5 and centrifuged. The supernatant was then transferred to a 100 mg Dowex 50 Na^+ column, previously treated with 10 ml M sodium acetate–acetic acid buffer, pH 6·5 and 5 ml redistilled water. After passage of the sample the column was washed with 30 ml 0·06 M phosphate buffer, pH 6·6 and 5 ml redistilled water. By this procedure all DOPA was eliminated from the column and the amine could then be eluted with 6 ml N HCl. This fraction was evaporated to dryness on a steam bath and extracted twice with 2 ml acetone containing 1 ml 0·1 N HCl per 100 ml. The acetone extract was transferred to plates and evaporated

to dryness. The registration of radioactivity was performed in a Nuclear, Chicago, gas flow-counter. The eluate contained some radioactive impurities which resulted in a background value of about 40 cpm. One μg of dopamine formed from this batch of ^{14}C-DOPA corresponded to 5,000 cpm. The details of this method will be given in a subsequent paper.

RESULTS

The optimal pH for enzyme activity was found to vary with the substrate concentration (Fig. 1). While the pH optimum was found to be 7·2 in experiments with a substrate concentration of 5×10^{-6} M it was 6·4 with a concentration of 5×10^{-3} M.

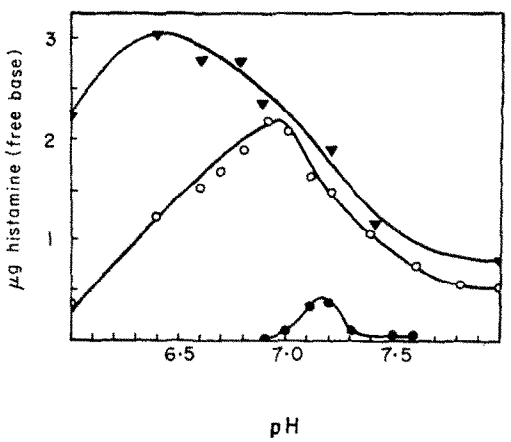


FIG. 1. The figure illustrates the change in "pH optimum" with different substrate concentrations. Enzyme activity is expressed as μg histamine formed. The conditions of the experiments are given in the text. At least three experiments were performed at each substrate concentration.

▼——▼ 5×10^{-3} M Histidine concentration, 10 μg pyridoxal-5-phosphate
○——○ 5×10^{-4} M Histidine concentration, 10 μg pyridoxal-5-phosphate
●——● 5×10^{-6} M Histidine concentration, 1 μg pyridoxal-5-phosphate.

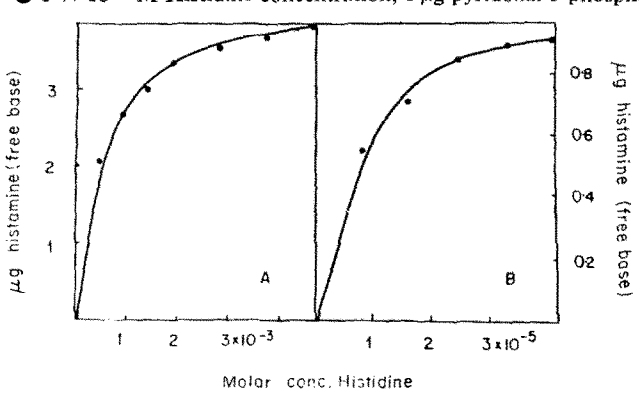


FIG. 2. The influence of pH on the substrate saturation of the enzyme.

A illustrates the relation between histamine formation and concentration of histidine at pH 6·4 and in the presence of 10 μg pyridoxal-5-phosphate.

B illustrates the relation between histamine formation and concentration of histidine at pH 7·6 and in the presence of 1 μg pyridoxal-5-phosphate. The results given in the figures represent only parts of the experiments. A further increase in the substrate concentrations did not result in higher enzyme activities. In some experiments performed at pH 7·6 higher concentrations of coenzyme was used with identical results.

The substrate saturation point of the enzyme turned out to be highly dependent on pH as shown in Fig. 2. At pH 7·6–8·0, a low Km value was observed; in more acid solution Km was markedly increased (Fig. 6).

Figure 3A shows the influence of time on histamine formation at pH 6·9 and at a histidine concentration of 5×10^{-4} M. Figure 3B illustrates the effect of enzyme concentration on activity under the same conditions. As is shown in these figures enzyme activity varies linearly with both of these variables.

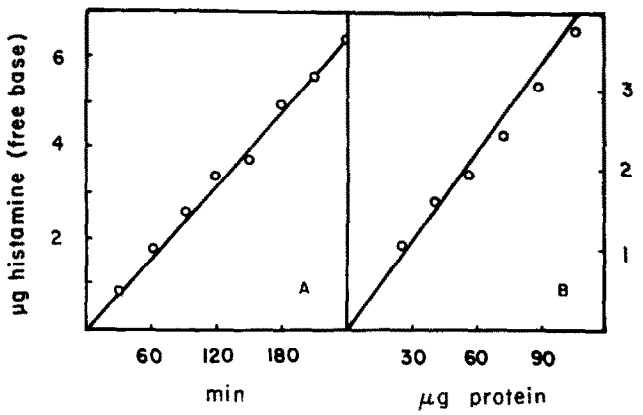


FIG. 3. The influence of incubation time and enzyme concentration on the rate of histamine formation. The incubations were performed at pH 6·9, at a substrate concentration of 5×10^{-4} M and in the presence of 10 µg pyridoxal-5-phosphate.

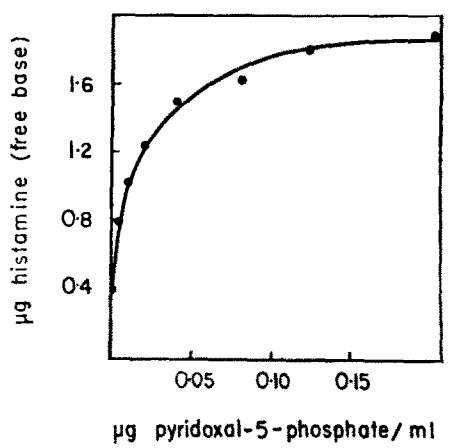


FIG. 4. The influence of coenzyme concentration on enzyme activity. The incubations were performed at pH 6·9 and at a substrate concentration of 5×10^{-4} M. The results given in the figure represent only a part of the experiment. A further increase in coenzyme concentration did not result in higher enzyme activity.

To obtain maximal enzyme activity pyridoxal-5-phosphate had to be added as shown in Fig. 4. It should be noted that very small amounts of coenzyme were needed to saturate the enzyme and that in all other experiments excess amounts were added.

Semicarbazide produced inhibition of the enzyme, probably by interacting with the coenzyme (Table 1). If more pyridoxal-5-phosphate was added the inhibition was reversed.

Even with high concentrations of L-DOPA (10^{-3} M) no significant inhibition of histamine formation was produced. When the enzyme was incubated with ^{14}C-DOPA at various pH in the presence of pyridoxal-5-phosphate no dopamine formation could be demonstrated.

TABLE 1. THE INFLUENCE OF PYRIDOXAL-5-PHOSPHATE ON SEMICARBAZIDE-PRODUCED INHIBITION OF ENZYME ACTIVITY

Semicarbazide molar conc.	Pyridoxal-5-phosphate molar conc.	Inhibition per cent.
0	6×10^{-5}	0
10^{-5}	6×10^{-5}	15
10^{-4}	6×10^{-5}	65
10^{-3}	6×10^{-5}	100
10^{-4}	6×10^{-5}	70
10^{-4}	4×10^{-4}	20
10^{-4}	6×10^{-4}	0

The table shows the results of one experiment performed at pH 6·9 and at a substrate concentration of 10^{-3} M.

DISCUSSION

Ganrot *et al.* reported a Km value for fetal histidine decarboxylase of 2×10^{-5} M at pH 7·8. On the other hand Burkhalter, working with the same enzyme found a Km of 10^{-3} M at pH 6·6, which he considered to be optimal. The results presented in this paper seem to imply that the discrepancy between Ganrot's and Burkhalter's observations is caused by different experimental conditions and reflects the true properties of the enzyme. The observed changes in pH optimum with increasing substrate concentrations and the corresponding change in Km values with different pH seem to offer a reasonable explanation of the differences between earlier results. Apparently the term "pH optimum" has little relevance for this enzyme unless the substrate concentration is stated. Likewise, it is obvious, that Km, to have any significance at all, must be strictly defined as regards pH. It is noteworthy that the Km value diminishes with increasing pH, indicating that the enzyme attacks a specific ionized form of histidine, which presumably occurs in higher concentrations at more alkaline pH. The isoelectric point of histidine is at pH 7·6. If the enzyme requires the zwitterion form, less of the latter will be available in acidic solution and more substrate must be added in order to saturate the enzyme. The results, however, seem to indicate that the enzyme requires the anionic form of the substrate rather than the zwitterion itself, since the apparent Km at pH 8·0 is rougly equal with that at pH 7·6. If the actual substrate were the zwitterion exclusively, the apparent Km would increase in both acid and basic solutions. The question therefore arises, whether it is possible from these observations to establish the exact nature of the ionized form of histidine which is attacked by the enzyme. Several, differently ionized, forms of histidine occur within the pH range in question. The concentration of each form at varying pH was calculated from the Henderson–Hasselbach equation (Fig. 5). The apparent Km was inversely related only to the percentage of histidine with an ionized anionic group and un-ionized imidazole and α-amino groups (Fig. 6). When the Km_{HID^-}-values, (HID^- representing the ionized form of histidine given in Fig. 6) were calculated from

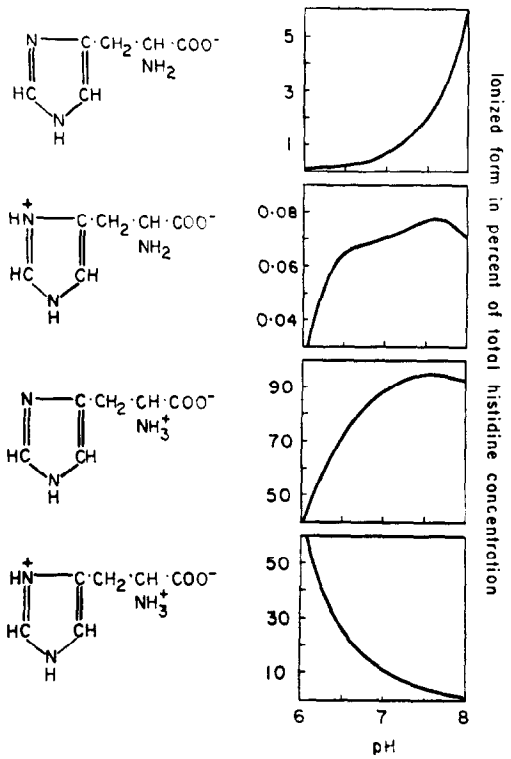


FIG 5. The influence of pH on the occurrence of some ionized forms of histidine. For further explanation see the text.

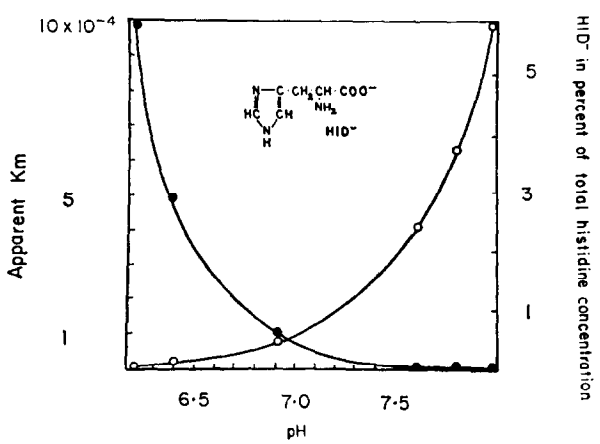


FIG. 6. The influence of pH on apparent Km and on concentration of HID^-. The nature of HID^- is illustrated in the figure. The Michaelis constant was estimated from experiments performed in principle as shown in Fig. 2. The results were plotted in the "double reciprocal" manner described by Lineweaver and Burk[11] and Km calculated according to their rearrangement of the Michaelis equation. For further explanation see text. At least two experiments were performed at each pH value.

the apparent Km and the known concentration of anionic histidine, approximately the same figure was obtained at each pH. The mean value was 6×10^{-7} M (range $4{\cdot}3$–$6{\cdot}9 \times 10^{-7}$). The ionization of the active group of the enzyme and its influence on the activity has not been taken into consideration in this discussion. From the experiments illustrated in Fig. 1 it may be concluded that the enzyme itself is affected by pH changes. In alkaline solution the activity was markedly reduced; indicating that the active form of the enzyme occurs more abundantly at acid pH. A further analysis of the ionization of the enzyme is in progress.

Pyridoxal-5-phosphate is a necessary cofactor. The presence of this agent is indispensable for enzyme activity. Preliminary experiments indicate that the enzymic requirement for pyridoxal-5-phosphate is influenced by several factors, such as pH, concentration of enzyme and concentration of substrate. This will be the purpose of a separate study. It is unlikely that these differences in coenzyme requirement has influenced the results presented here since excess amounts of pyridoxal-5-phosphate were added in all other experiments.

From experiments as shown in Table 1 it seems reasonable to suppose that semicarbazide inhibits enzyme activity by reacting with the cofactor.

Ganrot *et al.* suggested that the enzyme in the fetal rat acts as a specific histidine decarboxylase, thus being entirely different from the enzyme found in the rabbit kidney. The present results agree with this view. The enzyme preparation had no DOPA decarboxylase activity and large amounts of L-DOPA caused no significant reduction of enzyme activity. The histidine decarboxylase activity found in the kidney and the adult liver of different species has been attributed to an unspecific aromatic amino acid decarboxylase, supposed to be identical with DOPA decarboxylase.[12,13] The enzyme found in the fetal rat is obviously of another type. Certain tissues have been observed to develop a potent histamine forming capacity during the process of growth (cf. Kahlson). There are reasons to believe that the enzyme responsible for this activity is similar to or identical with fetal histidine decarboxylase.[14]

The significance of elevated histamine formation in various types of growing tissues has been much speculated upon. The role of histamine itself remains uncertain. The process of growth might however involve the development of an enzyme with certain characteristics similar to fetal histidine decarboxylase. The possibility exists that such an enzyme, apart from forming histamine, also takes part in the synthesis of other compounds necessary for the normal development of growing tissues.

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