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Histidine Decarboxylase of *Lactobacillus* 30a. IV. The Presence of Covalently Bound Pyruvate as the Prosthetic Group*

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ABSTRACT: Histidine decarboxylase from *Lactobacillus* 30a does not require pyridoxal phosphate for activity but is inhibited by reduction with sodium borohydride or by incubation with phenylhydrazine. In the latter reaction, 5 moles of [^{14}C]phenylhydrazine react/mole of enzyme to form a protein phenylhydrazone. This reaction is prevented by preliminary reduction with sodium borohydride, and also by the substrate, L-histidine, but not by D-histidine. These findings indicate that a carbonyl group forms part of the active center of the enzyme. When the enzyme is reduced with tritiated sodium borohydride, [α-^{3}H]lactate can be isolated from hydrolysates of the reduced protein, indicating that the unreduced enzyme contains bound pyruvate. Proteolytic digestion of enzyme [^{14}C]phenylhydrazone gives rise to a stable radioactive phenylhydrazone which, on purification and acid hydrolysis, yields phenylalanine as the only amino acid in amounts equimolar with the label. Following hydrogenation over platinum and hydrolysis, alanine is formed in nearly equimolar amounts. These results again demonstrate the presence of pyruvate in the native enzyme and indicate that it is combined with a phenylalanine residue of the protein by an amide linkage. *N*-Pyruvoylphenylalanine, synthesized by nonenzymatic transamination of alanylphenylalanine with pyridoxal, was converted into its phenylhydrazone and proved identical with the phenylhydrazone isolated from histidine decarboxylase in chromatographic, electrophoretic, and spectral properties and also in its behavior following reduction over platinum. Incomplete evidence indicates that the five pyruvoylphenylalanine residues in histidine decarboxylase occupy the N terminus of five of the ten peptide chains of this enzyme. A mechanism for the participation of pyruvate in the decarboxylation of histidine similar to that suggested for the participation of pyridoxal phosphate in the catalytic action of other amino acid decarboxylases is discussed.

Histidine decarboxylases (histidine carboxy-lyase, EC 4.1.1.22) catalyze the decarboxylation of histidine to yield histamine and CO_2. One such enzyme has been isolated in pure form from *Lactobacillus* 30a and has been partially characterized (Rosenthaler *et al.*, 1965; Chang and Snell, 1968a,b). Its structure is unusual in that it is apparently composed of ten subunits, giving a total molecular weight of 190,000 (Chang and Snell, 1968b). Unlike other amino acid decarboxylases studied thus far, this bacterial histidine decarboxylase does not require pyridoxal phosphate as a cofactor (Rosenthaler *et al.*, 1965; Chang and Snell, 1968a). However, it is inhibited by carbonyl reagents, implying that a carbonyl group is involved in the active site. The work to be described here shows that pyruvic acid is covalently bound to the enzyme through amide linkage with the amino group of a phenylalanyl residue, probably at the N terminus of a polypeptide chain. It is postulated that the pyruvoyl residue participates as a cofactor in the decarboxylation reaction in a manner analogous to the participation of pyridoxal phosphate in the enzymatic decarboxylation of other amino acids.

Methods

Histidine decarboxylase was purified as described by Chang and Snell (1968a) with the following slight modifications. Because of the relatively small preparative scale used, it was more convenient to employ the ratios and volumes described by Rosenthaler *et al.* (1965). After the heat step, the enzyme was collected by precipitation with ammonium sulfate (75% saturation) and the resulting concentrated fraction (*ca.* 10 ml) was clarified by centrifugation and then applied directly on the Sephadex G-200 column, omitting the acetone fractionation entirely. After recrystallization, the final material was indistinguishable from the preparation described by Chang and Snell (1968a) on the basis of ultracentrifugation, disc gel electrophoresis, and specific activity. Histidine decarboxylase activity was measured manometrically (Chang and Snell, 1968a). The concentration of protein in solutions of the pure enzyme was

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measured spectrophotometrically at 280 mμ. The extinction coefficient for the protein was determined in the following two ways. (1) Purified enzyme was dialyzed thoroughly, first against 0.1 M KCl and then against water. An aliquot of this solution, containing approximately 9 mg of protein, was dried to constant weight at 104° and the absorbance of the solution at 280 mμ was also determined by dilution of aliquots into 0.2 M ammonium acetate buffer (pH 4.8) and 0.05 M ammonium bicarbonate (pH 8.0). The average of two determinations gave extinction coefficients of 16.1 and 16.2 for a 1% solution of enzyme in ammonium acetate and ammonium bicarbonate, respectively. (2) The concentration of protein in a solution of histidine decarboxylase in 0.2 M ammonium acetate (pH 4.8) was first determined refractometrically in a Spinco Model E ultracentrifuge using a double-sector synthetic boundary cell and interference optics (Richards and Schachman, 1959) as recently described by Blethen *et al.* (1968). The extinction coefficient was then calculated from the measured absorbance of the solution at 280 mμ. A value of 16.2 was obtained in excellent agreement with the dry weight value. The average value (16.2) is 7% lower than that observed previously (Chang and Snell, 1968a). Protein concentrations determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Pentex) as a standard were generally 10–20% higher than the spectrophotometric values.

Reduction and carboxymethylation of histidine decarboxylase were carried out as described by Craven *et al.* (1965), except that the reaction was run in the presence of 5 M guanidine hydrochloride instead of 8 M urea.

Reaction of [1-^{14}C]phenylhydrazine with histidine decarboxylase was performed at a protein concentration of 0.5–6.0 mg/ml in 0.16 M ammonium acetate buffer (pH 4.8) and either 4.0 or 4.5 mM [1-^{14}C]phenylhydrazine hydrochloride (Tracerlab, 1.26 mCi/mmole, sometimes diluted with unlabeled phenylhydrazine hydrochloride (Eastman)). Incubations were done at 37° for 30–60 min unless otherwise indicated, although reaction was complete in less than 5 min. The reaction mixture (0.3–2.7 ml) was then applied to a column (0.8 × 25 or 1.7 × 26 cm) of Sephadex G-25 (coarse) equilibrated in 0.05 M ammonium bicarbonate; this procedure afforded good separation of the product from excess reagent. The protein phenylhydrazone eluted from the column was stable in ammonium bicarbonate for several weeks. When carboxymethylated histidine decarboxylase was treated with phenylhydrazine, the reaction mixture was made 3.2 M in guanidine hydrochloride to solubilize the carboxymethylated protein at the acidic pH (4.8). The extinction coefficient for the protein phenylhydrazone was estimated as follows. A reaction mixture (0.5 ml) containing 3.0 mg of protein was passed over a small Sephadex G-25 column, the fractions containing protein were combined, and the absorbance was measured at 280 mμ. Assuming that all of the protein applied to the column was eluted, an extinction coefficient of 18.5 for a 1% solution was calculated. This value was in fair agreement with the value (18.0) arrived at by correcting the extinction coefficient of the native enzyme for the additional absorbance at 280 mμ due to the phenylhydrazone. This latter correction was estimated by assuming that the extinction coefficient at 323 mμ of the protein phenylhydrazone and the pure model compound, pyruvoylphenylalanine phenylhydrazone (see Results), were the same per phenylhydrazone residue present, and then correcting for the absorbance of the latter compound at 280 mμ. An average value of 18.2 was adopted and used to determine concentrations of the phenylhydrazone derivative of histidine decarboxylase.

Reduction of histidine decarboxylase with sodium borohydride was initiated by the addition of one volume of a freshly prepared solution of 0.2 M $NaBH_4$ (Metal Hydrides, Inc.) or sodium borotritide (Nuclear-Chicago) in 0.1 N NaOH to nine volumes of enzyme solution (0.5–5 mg/ml) in 0.05 M ammonium bicarbonate. The final pH was 8.9. After incubation at room temperature for 30–60 min, the reaction mixture (0.3–1.6 ml) was applied to a column (0.8 × 25 or 1.7 × 26 cm) of Sephadex G-25 (coarse), equilibrated in 0.05 M ammonium bicarbonate. If the elution profile showed incomplete separation of the reduced protein from unbound radioactivity, the tritiated protein was dialyzed against ammonium bicarbonate. When sodium borotritide (4.9 Ci/mmole) was used, a 0.2 M solution was prepared in dry pyridine and diluted appropriately with 0.2 M $NaBH_4$ in 0.1 N NaOH. A control experiment showed that the amount of pyridine carried over in the reaction mixture did not affect the enzymatic activity.

Detection of Zones following Chromatographic or Electrophoretic Separations. After drying, paper electropherograms or chromatograms were cut into strips and the spots containing radioactivity were detected on a Vanguard automatic chromatogram scanner (Model 880). Radioactive spots on thin-layer chromatograms (Eastman silica gel layers, containing a fluorescent indicator, on a plastic backing) were detected either as described above or by autoradiography with No-Screen X-Ray Film. Unlabeled lactic acid was localized on paper strips with a reversed sugar stain for organic acids as described by Michl (1959) or, on thin-layer chromatograms, by spraying with an ethanolic solution of bromocresol green (Brown and Hall, 1950). Pyridine nucleotide standards and unlabeled phenylhydrazones were detected by examination of the chromatograms under ultraviolet light. A spray reagent containing 2,4-dinitrophenylhydrazine (Stahl and Jork, 1965) was used to visualize keto acids on thin-layer chromatograms; the spots were more easily detected after subsequent spraying with 1 N NaOH.

Other Methods. High-voltage electrophoresis was performed on Whatman No. 1 paper on a flat-bed apparatus designed by Crestfield and Allen (1955). Amino acid analyses were carried out on a Beckman-Spinco Model 120B automatic amino acid analyzer (Spackman *et al.*, 1958) modified with high-sensitivity cuvets and an expanded range card. Radioactivity was measured quantitatively by liquid scintillation counting in Bray's (1960) solution. The phenylhydrazone of pyruvic acid was synthesized by incubating phenylhydrazine hydrochloride (5 mmoles) and sodium pyruvate (4.5 mmoles) in 14.5 ml of 0.062 M ammonium acetate buffer (pH 4.7)

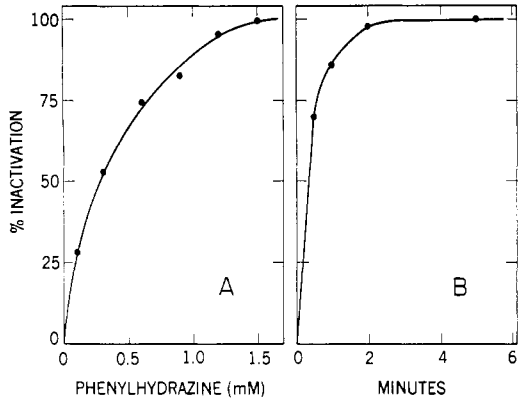

FIGURE 1: Inhibition of histidine decarboxylase. (A) By phenylhydrazine. Enzyme (9.6 μg) was assayed manometrically by tipping the enzyme solution in the side arm of a Warburg vessel into the main compartment containing buffer, substrate, and the indicated concentrations of phenylhydrazine. (B) By sodium borohydride. Enzyme (0.59 mg) was incubated in 0.3 ml of reaction mixture with 0.02 M sodium borohydride as described in Methods. Aliquots (20 μl) were withdrawn at intervals, diluted into 0.18 ml of 0.2 M ammonium acetate–0.1% bovine serum albumin (pH 4.8), and assayed manometrically.

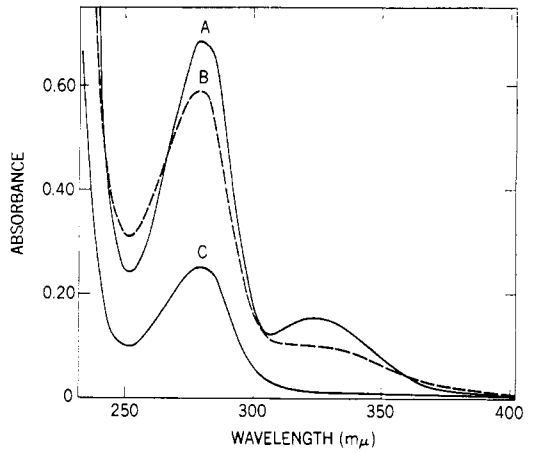

FIGURE 2: Spectra of histidine decarboxylase phenylhydrazone (A), carboxymethylated histidine decarboxylase phenylhydrazone (B), and sodium borohydride reduced histidine decarboxylase treated with phenylhydrazine (C) in 0.05 M ammonium bicarbonate. The protein concentrations were 0.38, 0.32, and 0.16 mg/ml, respectively, and the spectra were recorded on a Cary recording spectrophotometer (Model 14) in 1-cm quartz cuvets.

at room temperature for about 20 min. The product precipitated and was recrystallized several times from hot ethanol.

Results

Inhibition of Histidine Decarboxylase by Carbonyl Reagents. The catalytic action of histidine decarboxylase is inhibited by relatively high concentrations of phenylhydrazine (Figure 1A); the effect is maximal around 1.5 mM.

Incubation of the decarboxylase with sodium borohydride at slightly alkaline pH irreversibly inactivates the enzyme (Figure 1B). The reaction is complete in less than 5 min. Controls incubated without borohydride showed no loss of enzymatic activity even after 30 min under otherwise identical conditions.

Reaction of [1-^{14}C]Phenylhydrazine with Histidine Decarboxylase. Reaction of [1-^{14}C]phenylhydrazine with histidine decarboxylase (see Methods) in buffer at the optimum pH for this enzyme (pH 4.8) leads to the incorporation of approximately 5 moles of labeled phenylhydrazine/mole of enzyme. After separation from excess phenylhydrazine by passage over Sephadex, the product shows absorption maxima at both 280 and 323 mμ (Figure 2, curve A) as expected for a protein phenylhydrazone derivative. The ratio of absorbance at 323 to 280 mμ is an indication of the amount of phenylhydrazone formed and increases with increasing incorporation of phenylhydrazine to a maximum of 5 moles/mole of enzyme.

On carboxymethylation, histidine decarboxylase is dissociated to ten identical or very similar subunits (Chang and Snell, 1968b). The dissociated enzyme also reacts with phenylhydrazine in the presence of 3.2 M guanidine hydrochloride to give a product with a spectrum (Figure 2, curve B) similar to that of the phenylhydrazine-treated native enzyme. The magnitude of the absorbance at 323 mμ indicates that no additional phenylhydrazine has been bound as a phenylhydrazone. However, varying amounts of radioactivity (up to 12.5 molar equiv) in excess of 5 moles/mole of enzyme were bound to the carboxymethylated protein after passage over Sephadex. Unlike the phenylhydrazine bound specifically as the phenylhydrazone, which gives rise to the spectral band at 323 mμ, this nonspecifically bound radioactivity could be partially removed on dialysis. The nature of its binding is not understood; bovine serum albumin was also observed to bind phenylhydrazine in this manner.

To determine whether phenylhydrazine and sodium borohydride reacted with the same site on the protein, the enzyme was first reduced, dialyzed against 0.2 M ammonium acetate buffer (pH 4.8), and then treated with [1-^{14}C]phenylhydrazine. The final product gave a spectrum (Figure 2, curve C) which lacked the absorption maximum at 323 mμ due to the phenylhydrazone, but contained a variable amount of nonspecifically bound radioactivity after passage over Sephadex.

That the site reacting specifically with phenylhydrazine and with sodium borohydride is also that which reacts with L-histidine is indicated by the data of Table I. Under conditions such that nearly maximal phenylhydrazone formation occurs in the control, L-histidine largely prevents reaction of the enzyme with phenylhydrazine whereas D-histidine (which cannot act as a substrate (Rosenthaler *et al.*, 1965)) is very much less effective.

Identification of Lactate in Hydrolysates of Sodium Borotritide Treated Histidine Decarboxylase. To permit identification of the residue on the enzyme which reacts with $NaBH_4$, histidine decarboxylase was inactivated by reduction with [^{3}H]$NaBH_4$. The tritiated enzyme (8 mg) was dried from water several times and then hydrolyzed in 2 ml of constant-boiling HCl at 105° for

TABLE I: Effect of L- and D-Histidine on the Reaction of Histidine Decarboxylase with Phenylhydrazine.[a]

Addition (M)	$A_{323}:A_{280}$	Moles of Phenylhydrazine Bound/Mole of Enzyme
None	0.244	4.4
L-Histidine (0.20)	0.033	0.8
L-Histidine (0.67)	0.026	0.3
D-Histidine (0.20)	0.223	4.3
D-Histidine (0.67)	0.172	3.2

[a] Enzyme was incubated with [^{14}C]phenylhydrazine (see Methods) at room temperature for 2.5 min in the presence and absence of L- and D-histidine at a protein concentration of 1.2 mg (6.3 nmoles)/ml. Relatively high concentrations of the amino acid were used in an effort to approach the ratio of histidine to enzyme employed in the enzymatic assay.

TABLE II: Recrystallization of the Tritiated *p*-Bromophenacyl Ester of Lactic Acid to Constant Specific Radioactivity.[a]

No. of Recrystzn	Solvent	% Yield[b]	cpm/mg
1	Benzene–hexane	(100)	832
2	Benzene–hexane	87	916
3	Benzene–hexane	73	979
4	Benzene–hexane	61	1020
5	Benzene–hexane	53	1030
6	Benzene–hexane	46	1010
7	Chloroform–hexane	31	1020

[a] An aliquot (50 μl) of the purified radioactive sample isolated from borotritide-reduced enzyme (see text) was added to 200 mg of unlabeled lithium DL-lactate in 1 ml of water and refluxed for 1 hr with 200 mg of *p*-bromophenacyl bromide in 2 ml of 95% ethanol. Crude *p*-bromophenacyl ester was precipitated by addition of water and then recrystallized several times from the indicated solvents. [b] Data are calculated in terms of the 117 mg of crystals obtained from recrystallization 1, corrected for aliquots taken for analysis.

11 hr in the absence of air. The hydrolysate was dried over NaOH under vacuum and the residue was dissolved in 0.5 ml of 0.2 M pyridine acetate buffer (pH 3.1). This solution was applied to a Dowex 50 column (1.2 × 7.5 cm) which was equilibrated and eluted with the same buffer. Fractions (1.5 ml) containing radioactivity were pooled, dried, and dissolved in 1 ml of water. Approximately 90% of the radioactivity applied to the Dowex column was recovered in this fraction.

Aliquots (10 μl) of the radioactive fraction were subjected to thin-layer chromatography on silica gel with propanol–ammonia (3:2) as the developing solvent system and also to paper electrophoresis at 1800 V for 2 hr at pH 3.3 (0.05 M ammonium formate). The single tritiated compound present behaved identically to a standard of DL-lactic acid in both cases.

To confirm the identity of the compound as lactic acid, a portion of the purified radioactive sample was added to carrier unlabeled lactate and converted into the *p*-bromophenacyl ester (Shriner and Fuson, 1948). On recrystallization of the ester several times from benzene–hexane and once from chloroform–hexane, a constant specific radioactivity was obtained (Table II) and the melting point of the product (113°) agreed well with the value (112.8°) reported for the *p*-bromophenacyl ester of lactic acid (Judefind and Reid, 1920).

Finally, 50 μl of the Dowex eluate was incubated at room temperature with DPN$^+$[1] and lactic dehydrogenase in glycine buffer containing hydrazine (Hohorst, 1963). The reaction was followed at 340 mμ and was complete in approximately 15 min. Fractionation of the reaction mixture on a column of Bio-Gel P-2 (Figure 3A) shows that partial separation of the radioactive components was obtained, the [^{3}H]DPNH appearing last, apparently because of the tendency of the resin to retard compounds of an aromatic nature (Schwartz *et al.*, 1965; Uziel and Cohn, 1965). Essentially all of the radioactivity present in pool I of Figure 3A migrated as lactic acid while that in pool II migrated principally as DPNH with some DPN$^+$ (Figure 3B). Since lactic dehydrogenase utilizes only L-lactate, the enzyme catalyzes the transfer of tritium from the α-carbon in L-lactate to DPN$^+$, thus forming labeled DPNH and leaving residual D-[^{3}H]lactate. Autoxidation of DPNH to DPN$^+$ is not stereospecific, and some radioactivity therefore appears also in DPN$^+$.

The evidence presented here firmly establishes the formation of [α-^{3}H]lactic acid by sodium borotritide reduction and hydrolysis of histidine decarboxylase. The unreduced enzyme must therefore contain bound pyruvate.

The Nature of the Pyruvate–Histidine Decarboxylase Bond. To establish the nature of the bond between pyruvate and histidine decarboxylase, the radioactive phenylhydrazone of the enzyme was prepared free of excess reagent in the usual manner and then digested with Pronase and/or chymotrypsin. The radioactive fragment obtained was purified by passage over Sephadex G-25, followed by extraction under mildly acidic conditions into ethyl acetate. The amino acid composition of the partially purified product was then determined before and after hydrogenation over a platinum catalyst. By analogy with the behavior of keto acid 2,4-dinitrophenylhydrazones (Meister and Abendschein, 1956), the latter procedure should cleave the =N—N bond of the phenylhydrazone to yield the amino acid residue (alanine) corresponding to the keto acid (pyruvate) present in the original protein. The experiment

[1] See *Biochemistry 5*, 1445 (1966).

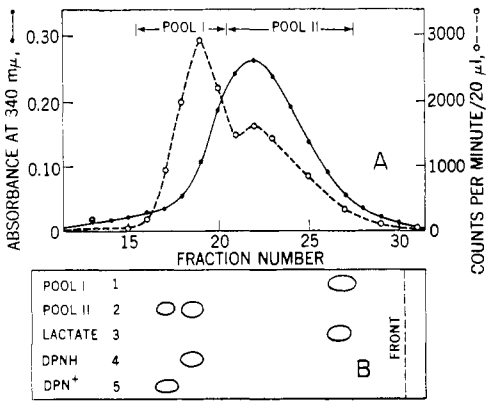

FIGURE 3: Chromatography of the products of reaction between lactic dehydrogenase, DPN^+, and [^{3}H]lactate isolated from tritiated histidine decarboxylase. (A) The lactic dehydrogenase reaction mixture (0.5 ml; see text) was applied to a column (0.8 × 45 cm) of Bio-Gel P-2, equilibrated and eluted with 0.1 M ammonium bicarbonate. The flow rate was 12 ml/hr. Each tube contained *ca.* 0.8 ml. Two separate pools (I and II) were made as indicated and lyophilized, and each was dissolved in 0.2 ml of water. (B) Results of ascending chromatography of the two samples on Whatman No. 1 paper for 21 hr with ethanol–1 M ammonium acetate (7:3; pH 7.5) as solvent. Aliquots of pool I, pool II, and standards of DL-lactic acid, DPNH, and DPN^+ were applied to points 1–5, respectively. Similar results (not illustrated) were obtained when pyridine–water (2:1) was the developing solvent.

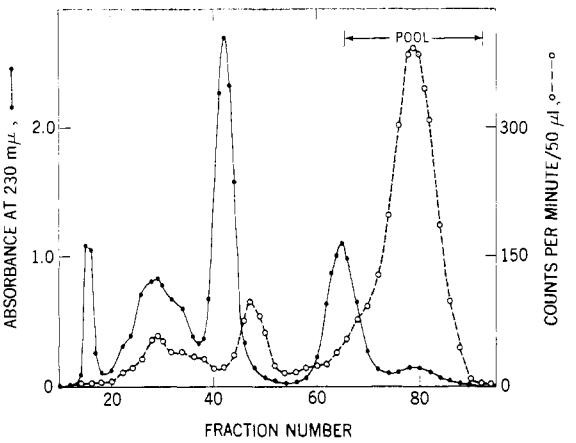

FIGURE 4: Chromatography of Pronase digest of histidine decarboxylase phenylhydrazone on Sephadex G-25. The concentrated digest (see text) was applied to a column (1.2 × 65 cm) of Sephadex G-25 (coarse), equilibrated, and eluted in the cold with 0.05 M ammonium bicarbonate. The flow rate was 10.2 ml/hr. Each tube contained *ca.* 1.7 ml.

reported below is representative of several which, although differing in minor respects, gave similar results.

A. ISOLATION OF A PHENYLHYDRAZONE FRAGMENT. Approximately 15 mg of the enzyme phenylhydrazone, which contained 5.0 moles of specifically bound phenylhydrazine/mole of protein, was dried and rinsed with water several times in a rotary evaporator. The residue was dissolved in 4 ml of 0.05 M ammonium bicarbonate–0.01 M calcium chloride and digestion was initiated by adding approximately 1 mg of Pronase. Additional Pronase (1 mg) was added at 3 and 12 hr. After a total of 20 hr at 37°, the reaction mixture was concentrated under vacuum to approximately 1 ml and fractionated over a column of Sephadex G-25. The elution profile (Figure 4) shows that extensive digestion had occurred and that most of the radioactivity was associated with a relatively small fragment which was retarded, and thereby partially purified, on the column. A pool of the radioactive material, containing about 74% of the radioactivity applied to the column, was made as indicated and dried under vacuum, and the residue was dissolved in 4 ml of 0.05 M ammonium bicarbonate. Approximately 1 mg of chymotrypsin was added and incubation was carried out for 3 hr at 37°. The digest was brought to dryness, dissolved in a small amount of water, and redried. This procedure was repeated once, the residue was dissolved in 4 ml of 0.2 M citrate buffer (pH 2.5), and this solution was immediately extracted three times with equal volumes of ethyl acetate. The combined extracts were evaporated under nitrogen. The final residue was dissolved in a small amount of ethyl acetate and subjected to analysis.

B. ANALYSIS OF THE PHENYLHYDRAZONE FRAGMENT BEFORE AND AFTER HYDROGENATION. An aliquot of the ethyl acetate solution, containing 35.6 nmoles of material on the basis of radioactivity, was evaporated to dryness, dissolved in 2 ml of constant-boiling HCl, and incubated at 105° for 14 hr in an evacuated and sealed tube. The HCl was then removed on a rotary evaporator and the sample was dissolved in 1.5 ml of 0.2 M citrate buffer (pH 2.2) and applied to the amino acid analyzer. A second aliquot containing the same amount of material was brought to dryness and, after mixing with 2 ml of water and 2 mg of PtO, was hydrogenated at 2.7–3.1 atm in a Parr hydrogenation apparatus at room temperature for 18 hr. The solution, which had been turbid at the start of the reaction, was completely clear upon allowing the reduced catalyst to settle. The supernatant solution was withdrawn after centrifugation and the residue was washed twice with small portions of water. The solutions were combined and evaporated to dryness; the residue was dissolved in 2 ml of constant-boiling HCl, and after removing a small aliquot for counting, was hydrolyzed as described above and subjected to amino acid analysis. The results (Table III) show that the phenylhydrazone before hydrogenation contains only phenylalanine in an amount equivalent to that subjected to analysis on the basis of its radioactivity, although small amounts of some other amino acid residues were also present. This observation suggests that pyruvate is covalently bound to a phenylalanyl residue in the peptide chain, presumably through an amide linkage. This hypothesis is supported by the appearance of nearly equimolar amounts of alanine on hydrogenation and subsequent hydrolysis of the phenylhydrazone. Phenylalanine was not found in the hydrogenated sample, presumably as a result of its hydrogenation to β-cyclohexylalanine (*cf.* Shemin and Herbst, 1938).

C. SYNTHESIS OF *N*-PYRUVOYLPHENYLALANINE AND ITS PHENYLHYDRAZONE. To confirm the structure of the phenylhydrazone indicated by the foregoing experiments, the isolated compound was compared with a synthetic sample of the phenylhydrazone of pyruvoylphenylalanine. Pyruvoylphenylalanine was prepared

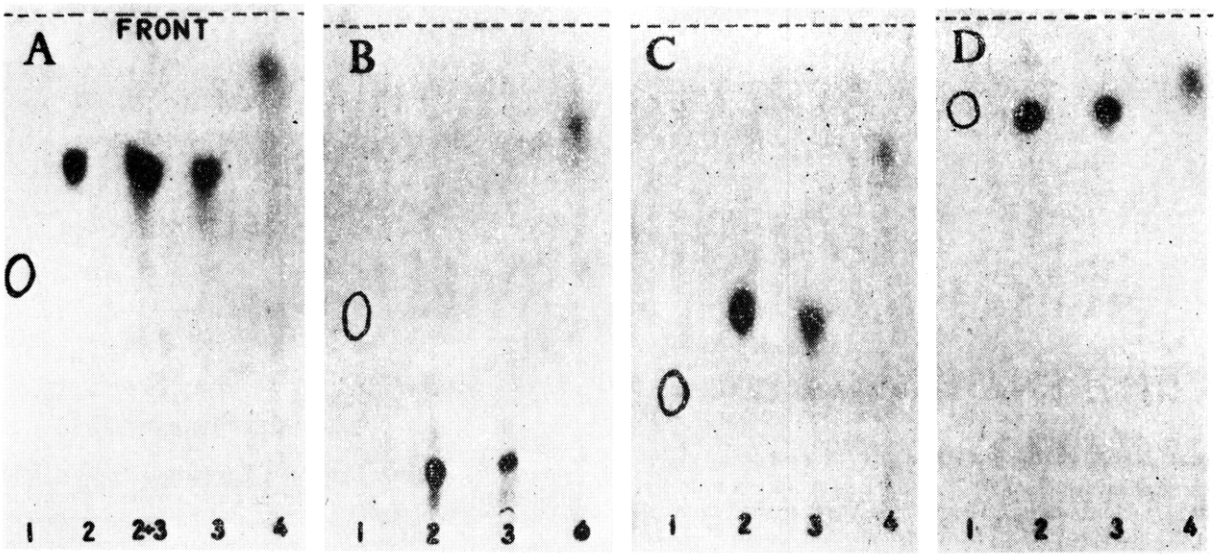

FIGURE 5: Chromatographic comparison of the radioactive phenylhydrazone isolated from proteolytic digests of histidine decarboxylase phenylhydrazone with *N*-pyruvoylphenylalanine phenylhydrazone. Pyruvate phenylhydrazone, pyruvolyphenylalanine phenylhydrazone, the phenylhydrazone from the enzyme, and phenylhydrazine hydrochloride were applied to the silica gel at points 1–4, respectively. In A, pyruvoylphenylalanine phenylhydrazone and the phenylhydrazone from the enzyme were also mixed before chromatography and applied at point (2 + 3). The developing solvent systems were: (A) methyl acetate–2-propanol–ammonia (9:7:4), (B) hexane–ethyl formate–propionic acid (2.6:1.4:0.3), (C) 1-butanol–ammonia (4:1), and (D) 1-butanol–acetic acid–pyridine–water (15:3:10:12). Pyruvate phenylhydrazone was detected under ultraviolet light and the other compounds by autoradiography. The photographs of the autoradiograms were made by direct contact printing on Fotorite paper.

TABLE III: Amino Acid Analysis before and after Hydrogenation of the Phenylhydrazone Purified from Proteolytic Digests of Histidine Decarboxylase Phenylhydrazone.

Amino Acid	Before Hydrogenation[a]		After Hydrogenation[b]	
	nmoles	equiv	nmoles	equiv
Phenylalanine	22.8	0.96	<0.7	<0.1
Alanine	*Ca.* 2	*Ca.* 0.1	16.2	0.87
All others	<4	<0.17	≤4	≤0.21

[a] The amount of phenylhydrazone applied to the acidic–neutral column of the amino acid analyzer was 23.7 nmoles. [b] The amount of phenylhydrazone applied to the column was 18.6 nmoles.

from alanylphenylalanine by nonenzymatic transamination with pyridoxal under the general conditions described by Metzler and Snell (1952). For this purpose, L-alanyl-L-phenylalanine (Mann Research Laboratories; 0.1 mmole), pyridoxal hydrochloride (0.5 mmole), and $AlNH_4(SO_4)_2 \cdot 12H_2O$ (0.01 mmole) in 10 ml of 0.1 M ammonium acetate buffer (pH 5.6) were heated at 100° for 1 hr. Concentrated HCl was then added to pH 1.1 and the mixture was fractionated on a Dowex 50 column (1.2 × 7.7 cm), equilibrated, and eluted with 20% formic acid. Fractions (*ca.* 2 ml) absorbing at 255 mμ were combined and lyophilized. The residue was dissolved in a small volume of water and the solution was again lyophilized. The product was dissolved in 2.4 ml of water and characterized by chromatography on silica gel layers before and after hydrolysis in constant-boiling HCl for 14 hr at 105°. With butanol–acetic acid–water (4:1:1) as developing solvent, a single ultraviolet-absorbing, ninhydrin-negative spot was observed before hydrolysis, which migrated more rapidly than either phenylalanine or pyruvate. In the hydrolyzed sample, this spot was absent, but two compounds corresponding exactly to phenylalanine and pyruvate in R_F values and chromogenic properties (with 2,4-dinitrophenylhydrazine and ninhydrin) appeared. The method of synthesis and the properties of the product indicate that it is *N*-pyruvoyl-L-phenylalanine.

To confirm the identity of this product, its phenylhydrazone was prepared as follows. The pyruvoyl phenylalanine solution (1 ml) was dried and the residue was redissolved in 0.8 ml of 0.2 M ammonium acetate buffer (pH 4.8). On incubation at 37° for 1 hr with 0.2 ml of 0.02 M [^{14}C]phenylhydrazine hydrochloride, the phenylhydrazone slowly precipitated. After washing with 0.2 M citrate buffer (pH 2.5), the residue was dissolved in a small amount of ethyl acetate. Thin-layer chromatography on silica gel with hexane–ethyl formate–propionic acid (2.6:1:4:0.3) as the developing

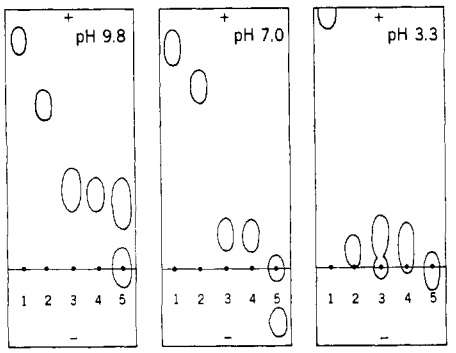

FIGURE 6: Diagrammatic respresentation of paper electropherograms of the phenylhydrazone isolated from proteolytic digests of histidine decarboxylase phenylhydrazone and *N*-pyruvoylphenylalanine phenylhydrazone. Picric acid, pyruvate phenylhydrazone, pyruvoylphenylalanine phenylhydrazone, the phenylhydrazone from the enzyme, and/or phenylhydrazine hydrochloride were applied at points 1–5, respectively. The buffers were 0.05 M ammonium carbonate (pH 9.8), 0.046 M acetate–0.068 M γ-collidine (pH 7.0), and 0.05 M ammonium formate (pH 3.3). Electrophoresis was conducted at 1800 V for 2 hr. Picric acid was detected visually, pyruvate phenylhydrazone under ultraviolet light, and the other compounds on the Vanguard chromatogram scanner.

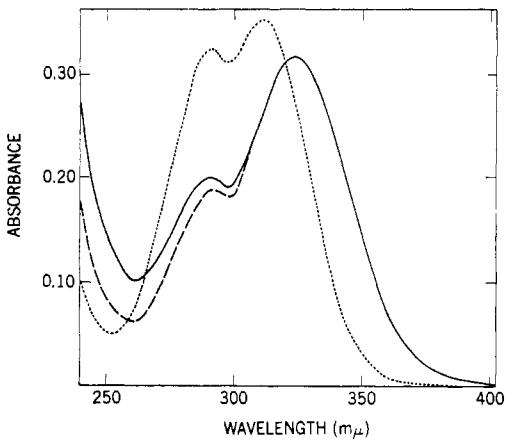

FIGURE 7: Spectra of the phenylhydrazone isolated from proteolytic digests of histidine decarboxylase phenylhydrazine (—) and pyruvoylphenylalanine phenylhydrazone (- - -). Aliquots of the two phenylhydrazone solutions were dried and the residues were dissolved in 0.05 M ammonium bicarbonate so that the final concentrations were 2.0×10^{-5} M on the basis of radioactivity in each case. The spectrum of pyruvate phenylhydrazone (· · ·; approximately 2.0×10^{-5} M) is included for comparison.

solvent showed a single radioactive spot which migrated more slowly than either phenylhydrazine or the phenylhydrazone of pyruvic acid. A similar sample, prepared with unlabeled phenylhydrazine and precipitated from ethanol with water several times, was dried and subjected to analysis. *Anal.* Calcd for $C_{17}H_{19}N_8O_3$: C, 66.44; H, 5.89; N, 12.92. Found: C, 66.37; H, 5.81; N, 13.14.

D. COMPARISON OF THE PHENYLHYDRAZONE FRAGMENT FROM HISTIDINE DECARBOXYLASE WITH THE PHENYLHYDRAZONE OF SYNTHETIC PYRUVOYLPHENYLALANINE. Samples (approximately 2.5 nmoles, 3.15 nCi) of the phenylhydrazone fragment from histidine decarboxylase were chromatographed on silica gel layers in four different solvent systems. In each case, over 90% of the radioactive material migrated as a single spot and cochromatographed with the synthetic pyruvoylphenylalanine phenylhydrazone (Figure 5). The compound was resolved from phenylhydrazine in all four systems and from pyruvic acid phenylhydrazone in three of them.

Similar samples were also subjected to high-voltage paper electrophoresis at pH 9.8, 7.0, and 3.3. At pH 9.8 and 7.0 the radioactive fragment from the enzyme digest migrated identically with the synthetic phenylhydrazone; at pH 3.3 both compounds partially decomposed but again showed similar distribution patterns of radioactivity (Figure 6).

The spectra of both phenylhydrazone solutions show maxima at the same positions (Figure 7), with identical absorbancies at 323 mμ. Minor differences in absorbancies occur at wavelengths below 305 mμ, perhaps reflecting the presence of minor impurities in the sample arising from the protein (*cf.* Table III).

Finally, the reference sample of phenylhydrazone, subjected to hydrogenation, hydrolysis, and amino acid analysis, gave rise to alanine, but phenylalanine was destroyed. The result duplicates that obtained with the phenylhydrazone isolated from the enzyme (see Table III).

The Position of Pyruvate in Histidine Decarboxylase. Pyruvate associated with D-proline reductase is probably bound through an amide linkage to a fragment which in turn is linked to the protein through an ester linkage (Hodgins and Abeles, 1967, and private communication). To determine whether a similar linkage is present in histidine decarboxylase, 0.73 mg of enzyme was tritiated by reduction with sodium borotritide, then heated at 100° in 0.1 N NaOH (0.5 ml) for 30 min, and finally passed over a column (0.8 × 27 cm) of Sephadex G-25 (coarse), equilibrated in 0.02 N NaOH. All of the radioactivity was eluted with the protein immediately after the void volume of the column. Thus, [^{3}H]lactate arising from bound pyruvate is not released as part of a small molecule on treatment with alkali sufficient to hydrolyze an ester linkage, but appears to be attached to the N-terminal residue of a polypeptide chain with a molecular weight greater than 5000, the exclusion limit of the resin.

Discussion

Although histidine decarboxylase does not require pyridoxal phosphate as a cofactor, it is inhibited by carbonyl reagents and is inactivated by borohydride reduction. The evidence presented here demonstrates that pyruvate in amide linkage to a phenylalanyl residue of the protein supplies the reactive carbonyl group and is apparently part of the active site of the enzyme. The latter conclusion is based on the observed inhibition of enzymatic activity by reaction with either sodium borohydride or phenylhydrazine and the fact that the substrate, L-histidine, protects against the binding of phenylhydrazine by the decarboxylase whereas D-histidine does not. In addition, unpublished kinetic experiments show that high concentrations of L-histidine

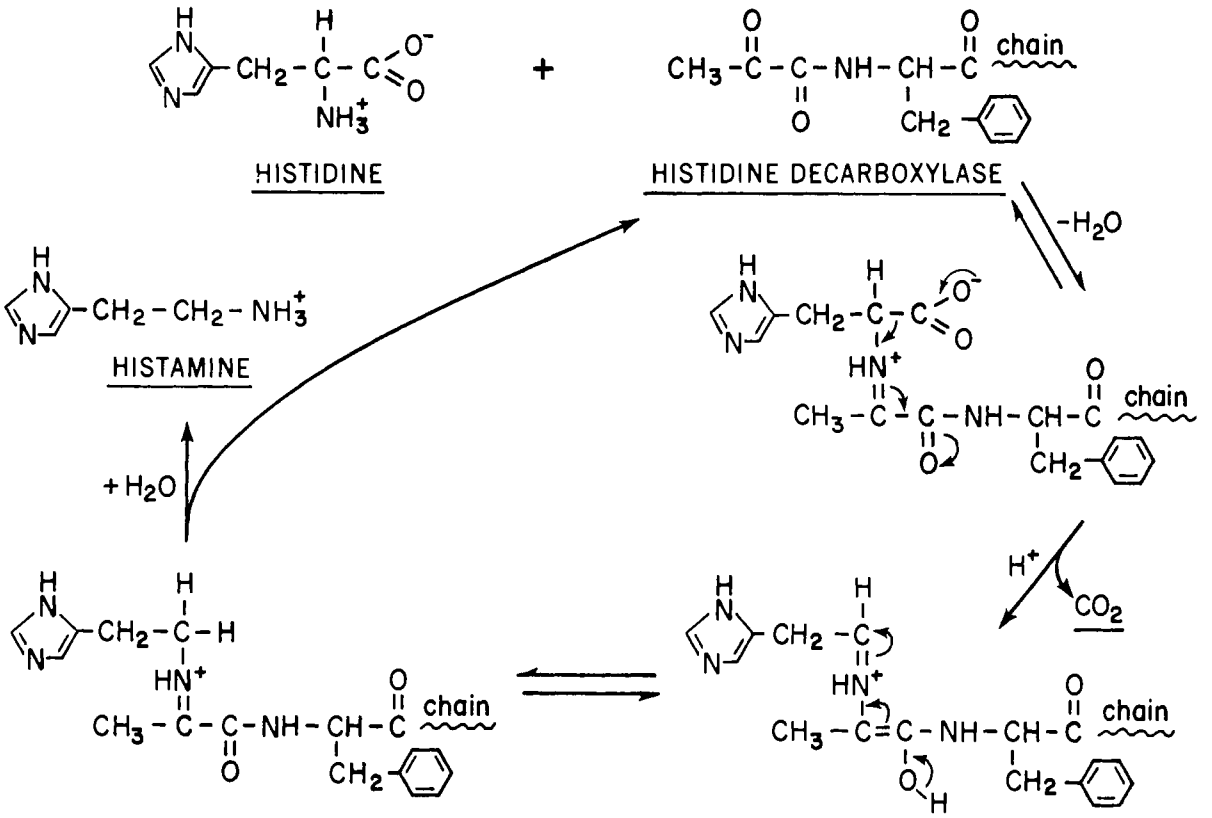

FIGURE 8: A suggested reaction mechanism for the participation of the pyruvoyl residue of histidine decarboxylase in the decarboxylation of histidine.

greatly decrease the rate of inactivation of histidine decarboxylase by phenylhydrazine. The interpretation of these experiments is complicated, however, by the irreversibility of the reaction between inhibitor and enzyme.

Spectral data indicate that the pure enzyme specifically binds 5 moles of phenylhydrazine (through phenylhydrazone formation) per mole of protein and therefore contains 5 moles of bound pyruvate. It is of interest to compare this observation with certain structural features of histidine decarboxylase. The enzyme is thought to be composed of ten identical or very similar subunits of mol wt 19,000, each containing a single cysteine residue; titration of five of the ten cysteine residues with *p*-chloromercuribenzoate completely inactivates the enzyme (Chang and Snell, 1968b). Similarly, only five of the ten carboxyl-terminal tyrosine residues are released on treatment of the native decarboxylase with carboxypeptidase A, with no concomitant loss of enzymatic activity (Chang and Snell, 1968b). All of these results are consistent with a model in which two monomers associate to form a dimeric basic catalytic unit of mol wt 38,000, as recently proposed by Chang and Snell (1968b). If, as suggested by the present work, only five of the polypeptide chains contain pyruvate, this provides direct evidence for the presence of two structurally different chains in the enzyme and supports the concept that one chain of each type might associate to form such a dimeric catalytic unit. The presence of two types of peptide chains, one terminating in pyruvate, would also help to explain the low yields of N-terminal serine reported earlier (Chang and Snell, 1968b). The presence of an active site involving the N terminus of a protein molecule is an unprecedented and unusual feature in enzyme structure and function and thus requires further study and confirmation. In D-proline reductase, which also appears to contain pyruvate as part of its active site (Hodgins and Abeles, 1967), pyruvate is apparently present in a side chain bound to the main peptide chain by an ester linkage. No evidence for such a linkage in histidine decarboxylase has so far been found; results of Sephadex filtration experiments would require that such a side chain, if present, approach the main chain in size.

The mechanism by which a pyruvoyl residue participates in enzymatic decarboxylation of histidine is not known. An attractive possibility (Figure 8) ascribes to the pyruvate in this enzyme a role similar to that played by pyridoxal phosphate in other amino acid decarboxylases (Metzler *et al.*, 1954a; Westheimer, 1960), or to glyoxylate in various nonenzymatic model reactions of amino acids (Nakada and Weinhouse, 1953; Metzler *et al.*, 1954b). The proposed mechanism assumes the presence of a rather highly electronegative oxygen in the amide linkage, perhaps induced by an appropriately placed group on the peptide chain with a pK value such as to assist in the postulated electron transfers *via* the transition, $>C{=}O + H{:}A\text{---} \rightleftarrows {=}\underset{|}{C}{-}OH + {:}A\text{---}$. Protonation of the amide nitrogen at the low pH optimum of histidine decarboxylase (pH 4.8) might also occur, thus enhancing the carbonyl character of the amide carbon. That pyruvate may not be as

TABLE IV: Comparison of Turnover Numbers of Different Homogeneous Amino Acid Decarboxylases.[a]

Enzyme	Source	Turnover No.[a]	Reference
Histidine decarboxylase	*Lactobacillus* 30a	2600	
Aspartic β-decarboxylase	*Achromobacter* sp.	5300	Wilson and Kornberg (1963)
Glutamic decarboxylase	*E. coli*	8300	Strausbauch and Fischer (1967)
Arginine decarboxylase	*E. coli*	34,800	Blethen *et al.* (1968)

[a] Turnover number is defined as the moles of product formed per minute per mole of bound coenzyme (pyruvate or pyridoxal phosphate).

efficient a cofactor as pyridoxal phosphate in the decarboxylase reaction is suggested by the comparison shown in Table IV. In terms of turnover number per bound prosthetic group (pyruvate or pyridoxal phosphate), histidine decarboxylase is only about half as effective as the least active pyridoxal phosphate dependent decarboxylase.

The hypothesis that pyruvate can participate as a prosthetic group in enzymic reactions requiring an active carbonyl function is now supported by two examples: histidine decarboxylase, studied here, and D-proline reductase, studied earlier by Hodgins and Abeles (1967). At least two additional enzymes, histidine deaminase (Smith *et al.*, 1967) and phenylalanine deaminase (Havir and Hanson, 1968), are known which do not contain pyridoxal phosphate but are inhibited by carbonyl reagents and which may, therefore, involve pyruvate or a functionally similar carbonyl compound.

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