4 W. L. HUGHES AND R. STRAESSLE, *J. Am. Chem. Soc.*, 72 (1950) 452.
5 J. F. RIORDAN AND B. L. VALLEE, *Biochemistry*, 2 (1963) 1460.
6 M. L. BENDER, J. R. WHITAKER AND F. MENGER, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 711.
7 C. F. JACOBSEN, J. LÉONIS, K. LINDERSTROM-LANG AND M. OTTESEN, in D. GLICK, *Methods of Biochemical Analysis*, Vol. IV, Interscience, New York, 1957.
8 T. L. COOMBS AND B. L. VALLEE, *Biochemistry*, 5 (1966) 3272.
9 I. SCHECHTER AND A. BERGER, *Biochemistry*, 5 (1966) 3371.

Received February 15th, 1967

Biochim. Biophys. Acta, 139 (1967) 211–214

PN 61132

Reversibility of the "irreversible" histidine ammonia-lyase reaction

The chemical reaction catalyzed by L-histidine ammonia-lyase (EC 4.3.1.3) is:

$$\underset{\text{L-histidine}}{R\text{–}CH_2\text{–}CH(NH_3^+)\text{–}COO^-} \rightleftharpoons \underset{\substack{\text{urocanate}\\(trans)}}{R\text{–}CH=CH\text{–}COO^-} + NH_4^+ \quad (R = \text{imidazole})$$

The reaction has been reported to be irreversible by WALKER AND SCHMIDT[1], MEHLER ANS TABOR[2], and PETERKOFSKY[3]. However, other well-known enzymes of this class catalyze ammonia eliminations that are readily reversible. For aspartate ammonia-lyase, K'_{eq} at 29° and pH 7.2 is 0.0074 (ref. 4); for β-methylaspartate ammonia-lyase, K'_{eq} is reported by BARKER *et al.*[5] to be 0.238 at 25° and pH 7.9. It appeared to us that the apparent irreversibility might result from the choice of unfavorable reaction conditions or insufficient incubation time.

Histidine ammonia-lyase was purified 125-fold from sonic lysates of *Pseudomonas fluorescens* A.3.12 by a modification of the method of PETERKOFSKY[3]. A protamine sulfate fractionation step was included; L-histidine was added during the heat step, and the enzyme was fractionated with $(NH_4)_2SO_4$ between 50–65% of saturation, rather than 0–50%. The final specific activity was 35 μmoles of urocanate per min per mg of protein. The assay system contained 0.1 M L-histidine (pH 8.0), 0.033 M Tris acetate (pH 8.0), 1 mM $MgSO_4$, 0.033 mM EDTA, 0.67 mM mercaptoethanol, and a suitable dilution of the enzyme.

The enzyme appeared to be homogeneous on gel electrophoresis. It had an approximate molecular weight of 198 000 as determined on a sucrose gradient including catalase as a reference.

In the histidine synthesis experiment we made the following departures from traditional systems[3,6]: (a) Tris acetate buffer was used rather than pyrophosphate because of the known sensitivity of the enzyme to metal-sequestering agents; (b) Mg^{2+} was added to ensure an adequate concentration of metal activator; and (c) the pH of the system was adjusted to 8.0 rather than 9.0–9.5, since PETERKOFSKY[3] has shown that the enzyme-catalyzed exchange of the β proton of histidine has an optimum pH near 7.5. The complete system contained the following: 0.5 M NH_4Cl (pH 8.0), 3.0 ml; 0.2 M potassium urocanate (pH 8.0), 3.0 ml; 0.01 M $MgSO_4$, 0.5 ml; 0.2 mg enzyme protein in 0.1 M Tris acetate buffer (pH 8.0), which was 1 mM in mercaptoethanol, 1.0 ml.

TABLE I

THE TIME COURSE OF THE REACTION AS MEASURED BY UROCANATE DISAPPEARANCE

Time (h)	$-\Delta A_{277m\mu}$ *(1:1000 dilution of incubation mixture)*	*Urocanate disappearance (μmoles/ml incubation mixture*)*
0	0.00	0.0
6	0.01	0.5
48	0.08	4.2
77	0.09	4.8
168	0.13	6.9

* Based on a molar absorbance for urocanic acid = 18 800 at 277 mμ.

The reaction course was followed spectrophotometrically at 277 mμ, where absorbance was due solely to urocanate and protein. The net decrease in absorbance with time is given in Table I. Extremely long times were required for the reaction to approach equilibrium; however, no bacterial invasion occurred. A solution identical with the incubation mixture except for the omission of enzyme was prepared as a control, in the event that a purely chemical synthesis might occur. No histidine could be detected in the latter.

The reaction mixture was tested periodically for the appearance of histidine by paper chromatography in 1-butanol–acetic acid–water (4:1:1, by vol.), and in 77% ethanol (by vol.). The first faint traces of ninhydrin-positive material were detectable after 22 h at room temperature. Only one ninhydrin-positive spot appeared and its intensity increased on day-to-day testing. Its R_F was identical with that of histidine. Duplicate paper chromatograms were sprayed with diazo reagent[7]. Both histidine and the newly synthesized substance (presumably histidine) gave orange-yellow spots with identical R_F values. The presence of histidine was further verified by electrophoresis on cellulose acetate. These data are given in Table II.

The new amino acid was identical with histidine in all tests and was definitely not glutamate. It is not possible that the single ninhydrin-positive spot could have arisen from enzyme decomposition during the long incubation period. The enzyme concentration was too low (about $1 \cdot 10^{-7}$ M) to give rise to a significant amount of

TABLE II

IDENTIFICATION OF HISTIDINE BY PAPER CHROMATOGRAPHY AND BY ELECTROPHORESIS

Solvent system No. 1: 1-butanol–acetic acid–water(4:1:1, by vol.) on Whatman No. 1 paper at 25°. Compounds detected by ninhydrin and diazo reagents. Solvent system No. 2: 77% ethanol on Whatman No. 1 paper at 25°. Compounds detected by ninhydrin and diazo reagents. Electrophoresis on cellulose acetate strip at pH 5.8 in 0.1 M Tris–maleate, 25°. 200 V applied for 15 min. Amino acids detected by ninhydrin.

Substance	R_F *in solvent system No. 1*	R_F *in solvent system No. 2*	*Distance moved (cm) toward cathode on electrophoresis*
L-Histidine (pH 8.0)	0.093	0.164	1.3
Amino acid from synthesis mixture (pH 8.0)	0.092	0.168	1.3

TABLE III

ESTIMATION OF K'_{eq} FOR HISTIDINE DEAMINATION: [UROCANATE] $[NH_4^+]$/[HISTIDINE]

	Final concentration of components (μmoles/ml)			K'_{eq}	*Time elapsed (h)*
	Urocanate	NH_4^+	*Histidine*		
Experiment I					
Based on urocanate disappearance	73	193	7	2	168
Experiment II					
Based on urocanate disappearance	75	195	5	3	168
Based on histidine formation	77	197	3	5	168
			Mean	3	

histidine, and histidine would not have been the sole product of decomposition.

The apparent equilibrium constant for the reaction was estimated from urocanate disappearance (spectrophotometrically) in two independent experiments and from histidine formation (quantitative assay by paper chromatography against standards) in the second of these two experiments (Table III). An average value of 3 was obtained for K'_{eq}, which is only an order of magnitude larger than K'_{eq} for the β-methylaspartate ammonia-lyase reaction at the same pH and temperature.

The establishment of the reversibility of the histidine ammonia-lyase reaction renders unnecessary the postulate of an obligate amino-enzyme intermediate in the reaction mechanism. Either a simple carbanion or concerted mechanism is consistent with the isotope-exchange data of PETERKOFSKY[3]. The rate-controlling step in the elimination reaction appears to be the dissociation of NH_3 from the enzyme active center. These aspects of the histidine ammonia-lyase reaction and comparable data for the β-methylaspartate ammonia-lyase reaction[8] have been discussed by ROSE[9] in a recent review. The rate-controlling step in the reverse reaction is not known, but may involve the binding of NH_3, since [^{14}C]urocanate is readily incorporated into histidine[3].

This research was supported by National Science Foundation Research Grant GB 5017.

Department of Biochemistry,
Louisiana State University,
Baton Rouge, La. (U.S.A.)

VIRGINIA R. WILLIAMS
JANICE M. HIROMS*

1 A. C. WALKER AND C. L. SCHMIDT, *Arch. Biochem. Biophys.*, 5 (1944) 445.
2 A. H. MEHLER AND H. TABOR, *J. Biol. Chem.*, 201 (1953) 775.
3 A. PETERKOFSKY, *J. Biol. Chem.*, 237 (1962) 787.
4 J. S. WILKINSON AND V. R. WILLIAMS, *Arch. Biochem. Biophys.*, 93 (1961) 80.
5 H. A. BARKER, R. D. SMYTH, R. M. WILSON AND H. WEISSBACH, *J. Biol. Chem.*, 234 (1959) 320.
6 H. TABOR AND A. H. MEHLER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 228.
7 H. TABOR, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 623.
8 H. J. BRIGHT, *J. Biol. Chem.*, 239 (1964) 2307.
9 I. A. ROSE, *Ann. Rev. Biochem.*, 35 (1966) 47.

Received February 6th, 1967

* Present address: Sinclair Oil Co., Houston, Texas, U.S.A.