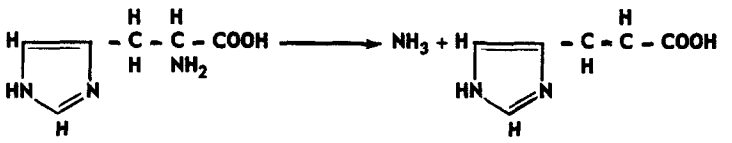
EVIDENCE FOR AMINO-ENZYME FORMATION DURING THE HISTIDASE REACTION

Alan Peterkofsky

National Institute of Arthritis & Metabolic Diseases
National Institutes of Health
Bethesda 14, Maryland

Received October 10, 1960

The histidase reaction is the first step in the major pathway of histidine degradation in mammalian tissues and microorganisms. The enzyme catalyzes the irreversible nonoxidative deamination of histidine to form urocanic acid:[1]



This study was designed to examine the possibility that the enzymatic process might be resolved into one or more partial reactions in which exchange of hydrogen, ammonia, or urocanic acid into the amino acid takes place. Evidence is presented in this communication which supports the following mechanism for the histidase reaction:

$$\text{Enzyme} + \text{histidine} \rightleftarrows \text{urocanic acid} + H^+ + \text{amino-enzyme} \rightarrow \text{enzyme} + NH_3 + \text{urocanic acid}$$

The adaptive enzyme from Pseudomonas was prepared by a modification of the method of Tabor and Mehler (1955) resulting in a purification of 60-80 fold. Table I indicates that, although urocanic acid does not react with free ammonia to form histidine (incubation #6) (Mehler and Tabor, 1953;

[1] For a review of the subject, see Tabor, 1954.

Yoshioka, 1955), histidase catalyzes an exchange of urocanic acid into histidine. These data indicate that an amino-enzyme exists as an intermediate in the histidase reaction.

Table I

Histidase-catalyzed Incorporation of C^{14}-urocanic Acid Into Histidine

	Incubation conditions	Time (mins.)	Urocanic acid at end of expt. (μmoles)	μmoles urocanic acid-C^{14} incorporated into histidine
1.	Complete system	0	5.3	.03
2.	Complete system	15	6.2	.24
3.	Complete system	60	8.3	.48
4.	Complete system minus enzyme	60	5.5	.02
5.	Complete system minus mercaptoethanol	60	7.3	.18
6.	Complete system minus histidine + ammonia	60	5.4	0

The complete system contained, in a total volume of 0.5 ml: sodium carbonate buffer, pH 9.2, 50 μmoles; histidine (sodium salt), 10 μmoles; mercaptoethanol, 10 μmoles; sodium urocanate-C^{14} (3280 cmp/μmole)*, 5 μmoles; histidase (80 x purified), 0.074 mg. Where indicated, 5 μmoles of NH_4Cl were added.

The reactions were terminated by the addition of 0.5 ml of 12% trichloroacetic acid. Residual histidine was purified on Dowex-50 H^+ and assayed colorimetrically by a modified diazo test (Tabor, 1957). Urocanic acid formation was estimated spectrophotometrically. (Mehler and Tabor. 1953).

*Urocanic acid-C^{14} was prepared biosynthetically by the action of histidase on 2-C^{14} (ring-labelled) histidine, followed by purification by column chromatography on Dowex-50 H^+. (Mehler et al., 1955).

If reversible amino-enzyme formation were characteristic of the histidase reaction, one would also expect a concurrent exchange of hydrogen ion between the medium and the β-carbon of histidine. Table II indicates that histidase catalyzes the incorporation of solvent tritium into a nonexchangeable form. The incorporation of tritium is dependent on mercaptoethanol, as is the conversion of histidine to urocanic acid. The irreversibility of the histidase reaction is further documented by the lack of tritium incorporation in the presence of urocanic acid and ammonia, when histidine is absent

(incubation #6); a net synthesis of histidine by reversal of histidase would necessarily result in the uptake of solvent tritium.

Table II

Incorporation of Solvent Tritium During Histidase Reaction in T_2O

	Incubation conditions	Time (mins.)	Urocanic acid at end of expt. (μmoles)	μatoms tritium incorporated
1.	Complete system	0	1.5	0.35
2.	Complete system	15	19	0.92
3.	Complete system	60	47	4.73
4.	Complete system minus enzyme + urocanic acid + NH_3	60	68	0.32
5.	Complete system minus mercaptoethanol	60	13	0.58
6.	Complete system minus histidine + urocanic acid + NH_3	60	61	0.23

The complete system contained in a total volume of 1.55 ml.: sodium carbonate buffer, pH 9.2, 100 μmoles; histidine (sodium salt), 100 μmoles; (except incub. #4, 50); mercaptoethanol, 15 μmoles; 4 mc T_2O; histidase (60 x purified), 0.1 mg. Where indicated, sodium urocanate (50 μmoles) and NH_4OH (50 μmoles) were included.

After incubation, the reaction mixtures were frozen and lyophilized, then treated with absolute ethanol to destroy the enzyme. Repeated addition of water and lyophilization were continued to remove residual exchangeable tritium. The dried samples were dissolved in a mixture of 2 ml. of 1 M hyamine hydroxide in methanol, 2 ml. of toluene containing 1.5% PPO* and .005% POPOP* and 13 ml. toluene. The samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314. The extent of urocanic acid formation at the end of the incubation period was estimated spectrophotometrically (Mehler and Tabor, 1953).

*Scintillation phosphors, obtained from Tracerlab Inc., Waltham, Mass.

Separation of the products of the reaction revealed that the tritium label was associated with histidine and not urocanic acid, suggesting a stereospecific tritiation of histidine. When enzymatically prepared tritium-labelled histidine was reincubated with histidase, the label was released from histidine at a rate essentially identical with that of urocanic acid

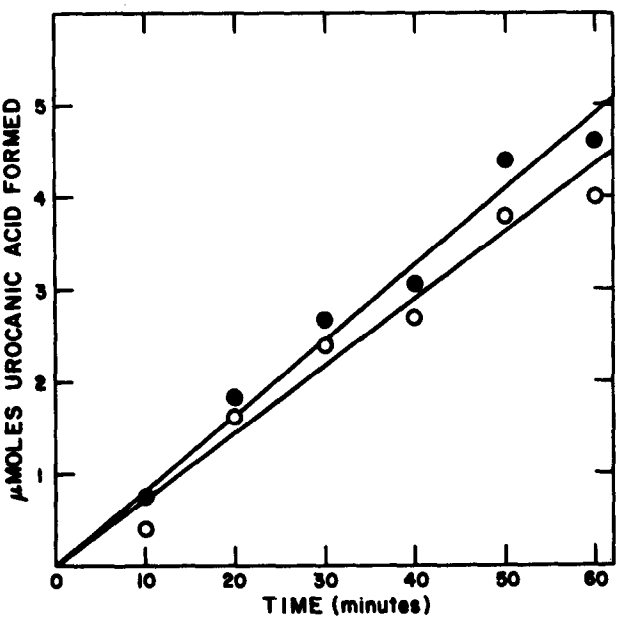


Fig. 1. Rate of detritiation of enzymatically labelled histidine. urocanic acid formation, measured spectrophotometrically; O, urocanic acid formation, measured by loss of non-exchangeable tritium from enzymatically-prepared labelled histidine.

Each incubation contained in a total volume of 1.4 ml.: enzymatically prepared tritium-labelled histidine*, 6 μmoles; sodium carbonate buffer, pH 9.2, 50 μmoles; mercaptoethanol, 10 μmoles; histidase (60 x purified), 38 μgms.

At the designated times, a sample was removed for the spectrophotometric determination of urocanic acid. The remainder of the incubations were treated as in Table II for termination of the reaction and assay of non-exchangeable tritium.

*A large scale histidase reaction was carried out in T_2O according to the conditions of Table II (complete system). The reaction was stopped when 50% of the histidine was converted to urocanic acid. The residual tritium-labelled histidine was isolated by descending paper chromatography in a t-butanol-formic acid-water solvent (Tabor, 1957).

---

formation (Figure 1). Since the detritiation due to the hydrogen exchange reaction occurs at only a small fraction of the rate of the complete reaction, one would not expect to detect a marked difference in the rate of tritium release compared to urocanic acid formation. These data support the idea that the hydrogen atom of histidine that undergoes the exchange reaction is the one that is removed from histidine in urocanic acid formation.

The localization of the radioactivity in enzymatically-prepared tritium-labelled histidine was achieved by a combination of chemical and enzymatic degradations to imidazoleacetic acid. The enzymatic degradation of histidine involved a preliminary treatment with histidine decarboxylase (Worthington Biochemical Company). The resultant histamine was essentially completely oxidized to imidazoleacetic acid by incubation with DPN and a high speed supernatant of a guinea pig kidney homogenate containing diamine oxidase and aldehyde dehydrogenase (Tabor, 1951; Weissbach et al., 1957). Essentially all of the label from tritiated histidine was retained in the imidazoleacetic acid. Thus, the exchanged hydrogen was not associated with the α-carbon of histidine.

In order to decide whether the labelled hydrogen was linked to the β-carbon of histidine or one of the imidazole ring carbons, labelled histidine was converted to cyanomethylimidazole by incubation for six hours with sodium hypochlorite, (Bauer and Tabor, 1957). The NaOH concentration was then adjusted to 0.3 N and incubation was continued overnight at room temperature. The long exposure of cyanomethylimidazole to alkaline conditions leads to chemical exchange of carbon-bound hydrogen adjacent to the cyano group (the β-carbon of histidine) (Reitz, 1939; Sprinson and Rittenberg, 1950). Subsequent hydrolysis of the nitrile (2 hours at 100°, 1 N NaOH) led to unlabelled imidazoleacetic acid. Although these data do not rigorously exclude the involvement of imidazole ring hydrogens in the exchange reaction they strongly suggest that the histidine was originally labelled in the β-position of the side chain. Thus, it appears that histidase carries out a specific hydrogen activation on the β-carbon of histidine.

The following lines of evidence suggest that the hydrogen exchange reaction is, in fact, a partial reaction of histidase and not due to a contaminating enzyme activity. Both the complete histidase reaction and the hydrogen exchange, as well as the urocanic exchange, are dependent on the addition of a sulphydryl compound in this 60-80 fold purified enzyme preparation. In addition, the hydrogen atom that participates in the exchange reaction seems to be the same one that is removed in the histidase-catalyzed deamina-

tion of histidine. Finally, kinetic studies indicate a similarity in the rate of hydrogen and urocanic acid exchange. This relationship appears to hold throughout the 50-fold purification of the enzyme.

When the histidase reaction was allowed to proceed in the presence of both T_2O and $N^{15}H_3$, no N^{15} was incorporated into the residual histidine under conditions where tritium underwent exchange. Thus, ammonia exchange does not occur.

The mechanism proposed is consistent with all the data presented here. A critical test has not yet been made to distinguish a difference in the rate of the two exchange reactions. This might allow a decision between a stepwise versus a "concerted" mechanism for amino-enzyme formation. In any case, it appears that an amino-enzyme exists as an intermediate.

I wish to thank Dr. H. Tabor for many helpful discussions.

REFERENCES

Bauer, H., and Tabor, H., Biochem. Preps., 5, 97 (1957).

Mehler, A. H., and Tabor, H., J. Biol. Chem., 201, 775 (1953).

Mehler, A. H., Tabor, H., and Hayaishi, O., Biochem. Preps., 4, 50 (1955).

Reitz, O., Z. physik. Chem. Abt. A, 183, 371 (1939).

Sprinson, D. B., and Rittenberg, D., J. Biol. Chem. 184, 405 (1950).

Tabor, H., J. Biol. Chem. 188, 125 (1951).

Tabor, H., Pharmacol. Revs., 6, 299 (1954).

Tabor, H., and Mehler, A. H., in S. P. Colowick and N. O. Kaplan (editors), Methods in Enzymology, Vol. II. Academic Press Inc., New York, (1955), p. 228.

Tabor, H., in S. P. Colowick and N. O. Kaplan (editors), Methods in Enzymology, Vol. III. Academic Press Inc., New York, (1957), p. 623.

Weissbach, H., Redfield, B. G., and Udenfriend, S., J. Biol. Chem., 229, 953 (1957).

Yoshioka, Y., Osaka Daigaku Igaku Zasshi, 7, 377 (1955).