SYNTHESIS OF HISTIDINE IN ESCHERICHIA COLI

II. RADIOISOTOPIC TRACER STUDIES*

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(Received for publication, July 5, 1955)

The precursor of L-histidine, L-histidinol, was first isolated by Vogel et al. (1) from cultures of an Escherichia coli mutant. More recently, L-histidinol, imidazole acetol, and imidazole glycerol have been found by Ames et al. (2, 3) in cultures of Neurospora mutants and have been implicated in the biosynthesis of histidine in Neurospora. These authors have shown that the phosphoric acid esters of the above imidazole alcohols are active intermediates in this synthesis, and they have suggested that pentose phosphate might be the precursor of the 5-carbon straight chain of the histidine molecule (4). Imidazole acetol has also been isolated from cultures of an E. coli mutant, and the normal biosynthetic pathway of histidine formation in that organism was shown to occur via histidinol and imidazole acetol.¹

The present paper reports the results of experiments designed to determine the pathway by which the 5-carbon chain of the histidine molecule is derived from glucose in E. coli. L-Histidinol synthesized by an appropriate E. coli mutant from glucose-1-C¹⁴ and glucose-6-C¹⁴ in separate experiments was isolated and chemically degraded to determine the distribution of C¹⁴ in the molecule. The data obtained indicate that the 5-carbon straight chain of histidine is derived from glucose exclusively via the glycolytic cycle and not at all by the hexose monophosphate shunt mechanism.

EXPERIMENTAL

Combustion and Assay of Radioactive Samples—Samples to be assayed were converted to CO₂ by the persulfate wet combustion procedure described by Calvin et al. (5). The CO₂ was collected as BaCO₃, suspended in methanol, and plated upon aluminum cups with a surface area of 3.47 sq. cm. The radioactivity of these samples was determined in a window-

* This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.
† Aided by a National Science Foundation Predoctoral Fellowship. Parts of this report appear in a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago, August, 1954. Present address, Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California.
¹ Westley, J., and Ceithaml, J., Arch. Biochem. and Biophys., in press.
less, gas flow counter with helium saturated with ethanol at 0° as the gas. Counting periods were sufficiently long to reduce counting errors to less than 5 per cent. Radioactivities were corrected to infinite thickness on the basis of the Reid self-absorption curve (6).

Sources of Isotopically Labeled Glucose—Glucose-6-C\textsuperscript{14} was synthesized by the method of Sowden (7). The radioactive purity of our product was tested by treating an aliquot of it with a 2-fold excess of sodium periodate. This reagent reacts with glucose to release carbon atom 6 as formaldehyde, while the other 5 carbon atoms are transformed into formate (8). After standing 1 hour at room temperature, the reaction mixture was made alkaline and was then steam-distilled. An aliquot of the distillate was analyzed quantitatively for formaldehyde (9), and the remainder was burned and counted as BaCO\textsubscript{3}. The radioactivity of the formaldehyde thus obtained accounted for 98 ± 4 per cent of the total radioactivity of the glucose-6-C\textsuperscript{14}.

Glucose-1-C\textsuperscript{14} was kindly supplied by Dr. Konrad Bloch. A sample of this preparation was degraded by oxidation with bromine to gluconic acid (10). Subsequent treatment of the gluconic acid with H\textsubscript{2}O\textsubscript{2} in the presence of ferric acetate liberated carbon 1 as CO\textsubscript{2}, while the remaining 5 carbon atoms became arabinose (11). Essentially all of the radioactivity was found in the CO\textsubscript{2} collected during the reaction, although some dilution of the specific radioactivity of carbon 1 did occur. The pentose produced in this reaction was obtained by deionizing the reaction mixture with Amberlite MB-3. This pentose contained less than 1 per cent of the radioactivity of the glucose-1-C\textsuperscript{14}.

Conditions of Incubation—Mutant CW-314 of E. coli strain W, which accumulates L-histidinol, was grown in ten to twelve individual cultures kept in 30 ml. screw top test-tubes. Each culture consisted of 15 ml. of minimal medium (12) containing unlabeled glucose (0.08 per cent) and supplemented with L-histidine (0.02 \textmu mole per ml.). This concentration of L-histidine limited the growth of the mutant to about 5 \times 10^8 cells per ml. The purpose of employing a series of individual cultures in place of one large one was to safeguard against a chance back-mutation to the wild type during the course of a radioactive experiment. Wild type cultures of E. coli not only do not accumulate L-histidinol, but can utilize it. No back-mutations occurred in any of our cultures during the course of our experiments.

After 24 hours of incubation with shaking at 37°, the bacterial suspensions were centrifuged and the cells were washed twice with minimal medium containing no glucose. The washed cells were then resuspended in minimal medium which contained as the sole utilizable carbon source 200 \gamma per ml. of either glucose-6-C\textsuperscript{14} or glucose-1-C\textsuperscript{14}, each possessing a specific activity of about 10^4 c.p.m. when counted as infinitely thick samples of
Following 24 hours of incubation with shaking at 37°, these resting cell suspensions were then chilled and centrifuged, and the supernatant fluids from the individual cultures were combined for the isolation of L-histidinol.

**Isolation of L-Histidinol**—The combined supernatant fluids containing L-histidinol were passed through a 1 X 10 inch column of Amberlite IRC-50 (H) which retained the L-histidinol. After the resin had been washed with distilled water, the L-histidinol was eluted with 0.1 N HCl. All fractions of the eluate giving a positive Pauly reaction (13) were pooled and distilled to dryness under reduced pressure. The dried residue was extracted with absolute ethanol, and this extract was evaporated to dryness with the aid of a stream of dry air. The L-histidinol dihydrochloride thus obtained weighed about 2 to 3 mg. and was dissolved in 10 ml. of water. Small aliquots of this solution were used for colorimetric analysis and for radioactive assay; the remainder was used for chemical degradation studies described in the next section. It is of interest to note that a 10 per cent radioactive yield of L-histidinol from glucose was obtained in these studies, i.e., 10 per cent of the total radioactivity present in the glucose originally added appeared in the L-histidinol finally isolated.

The colorimetric analysis for L-histidinol was carried out as follows. To each of a series of test-tubes were added 0.25 ml. of 1 per cent sulfanilic acid in 1 N HCl and 0.25 ml. of 0.5 per cent NaNO₂. Unknown samples containing an estimated 5 to 50 γ of L-histidinol dihydrochloride were added, followed by sufficient water to make the final volume in each tube 1.5 ml.; finally 0.5 ml. of 5 per cent Na₂CO₃ was added. The tubes were shaken and allowed to stand for 2 minutes, during which time an unstable red color developed. To each tube were then added 6 ml. of 1 M acetate buffer, pH 5.0. The yellow color which developed remained stable for several hours and was conveniently measured with a Coleman calorimeter against appropriate standards at 420 mμ.

To make certain that this procedure for the isolation of pure L-histidinol was effective, a preliminary experiment was performed in which unlabeled synthetic L-histidinol was added to the combined supernatant fluids containing radioactive L-histidinol before this solution was passed through the Amberlite IRC-50 (H) column. Subsequently, the L-histidinol in each fraction of eluate from the column which gave a positive Pauly reaction was isolated separately. The radioactivity of the L-histidinol isolated from each such fraction was determined by plating a sample of less than 1 mg. on an aluminum cup and counting it directly. With such thin samples, self-absorption may be neglected, and the radioactivity is proportional to the weight of the sample. The quantity of L-histidinol in each fraction was determined by the colorimetric method.

It was found that the L-histidinol obtained from each fraction of eluate
was equally radioactive in terms of counts per minute per mg. of L-histidinol. Moreover, when the L-histidinol from the richest fraction was isolated and recrystallized several times, there was no change in its radioactivity. The melting point of this crystalline material was identical with that of pure L-histidinol dihydrochloride.

In addition the various L-histidinol samples from this preliminary experiment were pooled and treated with sodium periodate to yield formaldehyde and imidazole acetaldehyde. The latter compound was then isolated by ion exchange procedures, and each fraction of the eluate containing imidazole acetaldehyde was worked up separately. As in the case of L-histidinol, the imidazole acetaldehyde in each fraction was found to contain the same specific radioactivity, although the amounts of the aldehyde in different fractions varied greatly. Moreover, the radioactivity of the pooled L-histidinol samples could be completely accounted for in the formaldehyde and imidazole acetaldehyde samples isolated, demonstrating that L-histidinol was the sole source of the radioactivity.

These findings proved the adequacy of the procedure for the isolation of L-histidinol from the incubation medium. In all experiments other than the preliminary one just described, no carrier L-histidinol was added prior to isolation, and all of the fractions of eluate containing L-histidinol were pooled before distillation to dryness and extraction with ethanol.

Chemical Degradation of Radioactive L-Histidinol—The degradation procedure for L-histidinol is outlined in Fig. 1. For these studies, the 2 to 3 mg. of L-histidinol dihydrochloride isolated from the incubation medium and dissolved in 10 ml. of water were diluted with 200 mg. of synthetic L-histidinol dihydrochloride. One-fourth of this diluted L-histidinol preparation was dissolved in 4 ml. of 2 N NaOH and shaken with three 0.2 ml. portions of benzoyl chloride to yield formate and the insoluble tetrabenzoyl compound. After removal of the precipitated tetrabenzoyl compound, the formic acid was steam-distilled from the acidified reaction mixture and oxidized to CO₂ with HgO (14). A sample of the tetrabenzoyl compound was also converted to CO₂ by the usual persulfate wet combustion procedure.

The remaining three-fourths of the diluted L-histidinol preparation was treated with an excess of NaIO₄ to yield imidazole acetaldehyde and formaldehyde. The formaldehyde was steam-distilled from the reaction mixture, determined colorimetrically (9), and also burned to CO₂. Imidazole acetaldehyde was also isolated.

It was found that imidazole acetaldehyde would not readily undergo the benzoylation reaction which results in rupture of the ring of most imidazole compounds (15). Imidazole ethanol, however, undergoes this

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Westley, J., and Ceithaml, J., to be published.
Fig. 1. Chemical degradation of L-histidinol. The numbers preceding or following CO\(_2\) refer to the L-histidinol carbon atoms they represent. The carbon atoms are numbered as shown in the structural formula for L-histidinol.
reaction readily. Therefore, one-half of the imidazole acetaldehyde isolated was reduced with sodium borohydride to imidazole ethanol. The product was isolated by passage of the acidified reaction mixture through a column of Amberlite IRC-50 (H) and the subsequent elution of the product with 0.1 N HCl.

The imidazole ethanol hydrochloride so isolated was then dissolved in 4 ml. of 2 N NaOH and shaken with three 0.2 ml. portions of benzoyl chloride to yield formic acid and an insoluble tribenzoyl compound. After removal of the precipitate, the formic acid was steam-distilled from the acidified solution and subsequently oxidized to CO₂ with HgO.

The tribenzoyl compound was hydrolyzed by refluxing for 2 hours in 2 N NaOH in 50 per cent ethanol. Although 2-keto, 4-hydroxybutylamine was not isolated, its presence in the hydrolysate, as indicated in Fig. 1, was expected since by an analogous reaction Shemin and Russell (16) obtained δ-aminolevulinic acid from an ester of imidazolepropionic acid. The presence of 2-keto, 4-hydroxybutylamine in the hydrolysate was verified by the production of formaldehyde and hydracrylic acid upon treatment of the hydrolysate with NaIO₄ in the following manner.

The hydrolysate was acidified and dried under reduced pressure. The residue was taken up in a small volume of water and extracted four times with an equal volume of ether each time to remove the benzoic acid. The aqueous solution was again dried under reduced pressure. The residue was dissolved in 20 ml. of water and 50 mg. of NaIO₄ were added. This solution was allowed to stand at room temperature for 1 hour, after which it was made slightly alkaline and was steam distilled. The steam distillate was found to contain formaldehyde, a portion of which was determined colorimetrically, while the remainder was burned to CO₂.

The alkaline periodate reaction mixture from which the formaldehyde had been removed was dried under reduced pressure, and the residue was first extracted with absolute ethanol to remove impurities and then boiled with 95 per cent ethanol to remove the sodium hydracrylate. This latter extract was dried under reduced pressure, and the residue was treated with a chronic acid reagent (17) which decarboxylates hydracrylic acid, yielding CO₂ and oxalic acid (18). After removal of sulfuric acid by treatment with Amberlite IR-4B (CO₃), the solution was concentrated under reduced pressure to 10 ml., and 1 ml. of 1 per cent CaCl₂ was added to precipitate calcium oxalate, which was collected, washed, and burned to CO₂.

The remaining half of the imidazole acetaldehyde preparation which had not been reduced with sodium borohydride was oxidized to imidazoleacetic

The sources of the several imidazole compounds used in the present investigation as authentic samples are described in Westley, J., and Ceithaml, J., Arch. Biochem. and Biophys., in press.
acid by refluxing briefly in concentrated HNO₃ (14). The solution was dried under reduced pressure, and the residue was dissolved in a small volume of water. Circular paper chromatograms (19) of this preparation, developed with 3:1 n-propanol-1 M acetic acid (20), showed imidazoleacetic acid (Rp:0.60) to be the sole imidazole compound present. The solution was then treated with Amberlite IR-4B (CO₃⁻) to remove nitric acid, after which the solution was concentrated to a small volume from which crystals of imidazoleacetic acid were obtained. These crystals were dissolved in a mixture of H₂SO₄ and redistilled chloroform and treated with sodium azide (21) to yield CO₂ and imidazole methylamine.

The H₂SO₄-chloroform reaction mixture was cautiously diluted with water and the chloroform removed with a stream of air. The addition of NaNO₂ to the diluted reaction mixture resulted in the deamination of imidazole methylamine to form 4(5)-hydroxymethylimidazole, which has an Rp value of 0.70 on circular paper chromatograms in the propanol-acetic acid system. The 4(5)-hydroxymethylimidazole was isolated by passing the reaction mixture through a column of Amberlite IRC-50 (H) and eluting the product with 0.1 N HCl. This compound was then oxidized with HNO₃ to imidazolecarboxylic acid, which was isolated by the method of Pyman (22).

The imidazolecarboxylic acid was heated above its melting point to yield CO₂ and a viscous distillate of imidazole which crystallized upon cooling (23). The imidazole was washed from the decarboxylation tube with a small volume of chloroform and reprecipitated by addition of petroleum ether. The crystals so obtained were dried in vacuo at 75° and then were burned to CO₂.

All CO₂ samples obtained from the above procedures were collected as BaCO₃ and plated for assay of their radioactivity.

RESULTS AND DISCUSSION

The specific radioactivities of the glucose employed, the L-histidinol isolated, and the various products obtained from the degradation of L-histidinol are reported in Table I as counts per minute of infinitely thick samples of BaCO₃. The total radioactivities of the degradation products of L-histidinol are also given, expressed in terms of percentages of the total radioactivity of L-histidinol isolated. The percentage of the total radioactivity of L-histidinol found in each of the individual carbon atoms is depicted in Fig. 2.

Glucose metabolism in E. coli is known to proceed by at least two separate pathways: (1) glycolysis and subsequent oxidation and (2) the hexose monophosphate shunt mechanism (24). These are indicated schematically for glucose-1-C¹⁴ and glucose-6-C¹⁴ in Fig. 3.
Synthesis of the 5-carbon chain of L-histidinol by condensation of glucose metabolites from Pathway 1 would result in a high degree of similarity in the data from the experiments in which glucose-1-C\textsuperscript{14} and glucose-6-C\textsuperscript{14} were used as carbon sources. In contrast, synthesis of L-histidinol directly from glucose by the shunt mechanism would result in great dissimilarity in such data.

**Table I**

*Distribution of C\textsuperscript{14} in L-Histidinol Synthesized from Glucose-6-C\textsuperscript{14} and Glucose-1-C\textsuperscript{14}*

<table>
<thead>
<tr>
<th>Sources of BaCO\textsubscript{3} assayed</th>
<th>Specific activities ( \times 10^{-3} )</th>
<th>Per cent of total radioactivity of L-histidinol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose-6-C\textsuperscript{14} experiment</td>
<td>Glucose-1-C\textsuperscript{14} experiment</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.5</td>
<td>8.04</td>
</tr>
<tr>
<td>C\textsubscript{6} of glucose (calculated)</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{1}</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>L-Histidinol</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>C\textsubscript{2} (L-Histidinol)</td>
<td>0.52</td>
<td>8.3</td>
</tr>
<tr>
<td>C\textsubscript{4}</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td>C\textsubscript{6}</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>C\textsubscript{7}</td>
<td>1.5</td>
<td>3.8</td>
</tr>
<tr>
<td>C\textsubscript{8}</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{6} and C\textsubscript{7} (oxalate)</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{8}, C\textsubscript{4}, and C\textsubscript{4} (imidazole)</td>
<td>4.8</td>
<td>8.0</td>
</tr>
<tr>
<td>C\textsubscript{4}, C\textsubscript{3}, C\textsubscript{7}, and C\textsubscript{8} (as tetra-benzoxy compound, containing 33 C atoms)</td>
<td>1.7</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The numbering system for L-histidinol is presented in Fig. 2.

The results reported in Table I and Fig. 2 indicate a synthesis of the 5-carbon chain by condensation of a 3-carbon unit (carbon atoms 4, 5, and 6 of L-histidinol) with a 2-carbon unit (carbon atoms 7 and 8 of L-histidinol) from pathway 1 of glucose metabolism. Synthesis of the L-histidinol chain directly from glucose by Pathway 2 is excluded by the high degree of similarity in the data from the two experiments. The only substantial difference between the labeling patterns obtained in the two experiments occurs in carbon atom 4. The higher degree of labeling in the glucose-1-C\textsuperscript{14} experiment could be attributed to the production of C\textsuperscript{14}O\textsubscript{2}.
and its subsequent fixation into the 3-carbon unit in the position corresponding to the carboxyl carbon of pyruvate.

![Diagram of glucose metabolism](image)

**Fig. 2.** Distribution of C\(^{14}\) in L-histidinol from glucose-6-C\(^{14}\) and from glucose-1-C\(^{14}\) expressed in terms of percentage of the total radioactivity of L-histidinol.

1. ![Diagram of glucose metabolism](image)

2. ![Diagram of glucose metabolism](image)

**Fig. 3.** Schematic representation of glucose-6-C\(^{14}\) and glucose-1-C\(^{14}\) metabolism via Pathway 1, glycolysis and subsequent oxidation and Pathway 2, the hexose monophosphate shunt mechanism.

Moreover, synthesis of the 5-carbon chain by both pathways concurrently is also excluded for the following reason. In the 3-carbon unit
arising in Pathway 1, there is at once a dilution of the specific activity of the labeled carbon atom to one-half of its value in the glucose used as substrate. If the 5-carbon chain of \( L \)-histidinol arises from both pathways of glucose metabolism, there should be a further dilution of the specific activity in the labeled position, at least in the experiment involving glucose-1-C\(_{14}\). The data of Table I show, however, that the specific activity of carbon atom 6 of \( L \)-histidinol synthesized from glucose-1-C\(_{14}\) is 50 per cent of that of carbon atom 1 of the glucose. In the other experiment, the specific activity of carbon atom 6 of \( L \)-histidinol synthesized from glucose-6-C\(_{14}\) is 46 per cent of that of carbon atom 6 of the glucose. These data support the view that the synthesis of the 5-carbon chain of \( L \)-histidinol proceeds by way of Pathway 1 exclusively.

From the results presented in Table I, however, the possible participation of pentose or pentose phosphate in the synthesis of \( L \)-histidinol in \( E. \ coli \), grown under varying conditions, cannot be conclusively excluded. Lanning and Cohen (25), using rapidly growing cultures under vigorous aeration, reported that pentose was synthesized in their system primarily, but not solely, by the shunt mechanism. Actually their data indicate that as much as 20 to 30 per cent of the pentose produced might have arisen by a condensation of 2- and 3-carbon units. The vigorous aeration which these authors employed would have favored the shunt mechanism by rapidly removing \( CO_2 \) from the culture medium. In contrast to the conditions employed by Lanning and Cohen, the present investigation was carried out with resting cell cultures aerated by shaking. It is conceivable that these conditions might favor pentose synthesis in \( E. \ coli \) by a condensation of 2- and 3-carbon units arising from the glycolytic pathway and subsequent oxidation rather than by way of the shunt mechanism. Moreover, it should be noted that, if the condensation of 2- and 3-carbon units to form the 5-carbon chain of the histidinol molecule in \( E. \ coli \) were the same as that which Bernstein (26) has demonstrated for pentose formation in chickens, then the \( L \)-histidinol should be labeled principally in the 4 and 8 or the 5 and 8 positions. This does not correspond with the results of the present work.

The results presented in Table I and Fig. 2 are consistent with the findings of Cutinelli et al. (27) that the carboxyl carbon of histidine obtained from \( E. \ coli \), grown on doubly labeled acetate as the sole carbon source, is derived from the methyl carbon of acetate. On the other hand the results are not in agreement with the data reported by Levy and Coon (28) for histidine synthesis from glucose-1-C\(_{14}\) in yeast.

**SUMMARY**

\( L \)-Histidinol synthesized by a mutant strain of *Escherichia coli* from glucose-6-C\(_{14}\) and glucose-1-C\(_{14}\) in separate experiments was isolated and chem-
ically degraded to determine the distribution of C¹⁴ in the molecule. The data obtained indicated that, in the aerobic resting cell system employed, the 5-carbon chain of L-histidinol was synthesized by a condensation of 2- and 3-carbon units arising from glycolysis and subsequent oxidation. Participation of the hexose monophosphate shunt mechanism in this synthesis was excluded.

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*J. Biol. Chem.* 1956, 219:139-149.

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