The formation of 5-amino-1-ribosyl-4-imidazolecarboxamide 5'-phosphate by bacterial extracts incubated with adenosine triphosphate, an ATP-generating system, ribose-5-P, and glutamine has been reported by Love (1) and by Gots and Gollub (2). We have been able to confirm these observations; in addition we find that a derivative of ACP, rather than ACP itself, is formed when glutamine is omitted (3). There is no evidence for the participation of such a compound in the sequence of reactions responsible for the synthesis de novo of the purine precursor, ACP (4). This finding suggested that the observed formation of ACP from ATP, ribose-5-P, and glutamine is the result of reactions other than those involved in the biosynthesis of purines. Such an interpretation is also supported by these additional observations: (a) Dialyzed bacterial extracts are able to produce ACP in the absence of added formate, CO₂, and glycine, compounds which are all necessary for its synthesis de novo (2). (b) Added glycine-2-C¹⁴ is not incorporated into the ACP produced. (c) ACP is produced by extracts of a mutant which lacks the ability to convert 5-amino-1-ribosylimidazole 5'-phosphate to ACP, an essential reaction in the biosynthesis of purines (1).

A possible source of the ACP produced under these circumstances is ATP itself. The hypothesis that ACP is indeed derived from ATP by removal of a carbon atom and a nitrogen atom was confirmed by the demonstration that added AMP-S-C¹⁴, which equilibrates readily with ATP, was incorporated into the ACP without dilution. Furthermore, earlier experiments in this (5, 6) and other laboratories (7, 8) had shown that bacteria are capable of obtaining the N₇-C₅ portion of the imidazole ring of histidine from carbon 2 and an attached nitrogen atom of purine bases provided in the growth medium. The possibility therefore had to be considered that the conversion of ATP to ACP was involved in the biosynthesis of the imidazole ring of histidine. This assumption was substantiated by the finding that in addition to ACP a histidine precursor, l-erythro-imidazoleglycerol phosphate ester, is a product of these reactions.

The nature of these enzymatic steps and the evidence for their role in histidine biosynthesis are the subject of this paper.

EXPERIMENTAL

Materials and Methods

Bacteria—Aerobacter aerogenes, strain 1033, and the guanine-requiring mutant, strain P-14, derived from it have been described previously (9). The histidine-requiring mutants of Salmonella typhimurium (strains, hi A-5, hi B-12, and hi F-41) were obtained from Dr. M. Demerec of the Long Island Biological Association. The methods of cultivation have been described previously (10).

Chemicals—Dipotassium ATP and AMP (Pabst Brewing Company); AMP-8-C¹⁴, adenine-2-C¹⁴, the barium salt of ribose-5-P, and glutathione (Schwartz Laboratories, Inc.); glutamine and protamine sulfate (Nutritional Biochemicals Corporation); and diliithium acetyl-P (Mann Research Laboratories, Inc.) were commercial preparations. Ribose-5-P-1-C¹⁴, imidazoleglycerol phosphate, and imidazoleformaldehyde were gifts of Dr. Bruce Ames.

Analytical Methods—AMP was identified and estimated by its ultraviolet absorption spectrum; imidazoleglycerol phosphate was estimated by its reaction with diazosulfanilic acid (11) or by oxidation to imidazoleformaldehyde with periodic acid (12); and ACP was estimated by its ultraviolet extinction at 267 mp (13) or by the test of Bratton and Marshall (14) for diazotizable amines.

Preparation of Extracts—Extracts³ were prepared by sonic oscillation of cell suspensions (5 g of packed cells per 25 ml of 0.03 m potassium phosphate buffer, pH 7.4) for 6 minutes in a 10 kc. magnetostrictive oscillator (Raytheon). Intact cells and large particles were removed by centrifugation for 15 minutes at 25,000 X g. The extracts were dialyzed for 16 hours against 0.03 m potassium phosphate buffer, pH 7.4. To each 100 ml of dialyzed extract, 12 ml of a 2% solution of protamine sulfate were added. The resulting precipitate was removed by centrifugation and discarded. The protein content of the treated extracts was usually 10 to 12 mg per ml.

Assay System—The reaction mixture contained per ml: ATP, 2.1 μmoles; ribose-5-P, 4.2 μmoles; acetyl-P, 16.3 μmoles; reduced glutathione, 12.6 μmoles; glutamine, 12.6 μmoles; tris-(hydroxymethyl)aminomethane, pH 8.06, 84 μmoles; MgCl₂, 16.8 μmoles; and 0.33 ml of treated extract. The volume of the reaction mixture for routine assays was 0.6 ml. After 15 minutes at 37° the reaction mixture was deproteinized by the addition of an equal volume of 10% trichloroacetic acid; the arylamine formed was determined by the method of Bratton and Marshall (14); the imidazole derivative formed was determined by its reaction with diazosulfanilic acid (11).

RESULTS

Formation of Arylamine and Imidazole—Extracts of several bacterial species acting on ATP, ribose-5-P, glutamine, and

* Extracts of the histidine requiring mutants were prepared from cells which had been grown in a mineral-salts glucose medium.
butyric acid-HzO-concentrated ammonium hydroxide (100 : 58 : 2). test (I3), and its RF in paper chromatography (13) with iso-

its ultraviolet absorption spectrum (13), the absorption spectrum acid (12). The arylamine was identified as ACP on the basis of

3: 1 (16). Furthermore, like imidazoleglycerol phosphate, it was completely from solution by passage over a column of Dowex 2 resin [Image 0x-19 to 579x792]systems, propanol-0.2 glycerol phosphate in paper chromatography with two solvent

HCl, AMP with 0.0025 Na acetate and ethanol according to the method of LePage (15). The arylamine and the imidazole appeared to be in the form of

acetate and ethanol to 100 ml of extract

The results summarized in Table II show that carbon 1 of ribose-5-P was incorporated into imidazoleglycerol phosphate without dilution. The imidazoleformaldehyde derived from imidazoleglycerol phosphate had 80% of the radioactivity of the parent compound. In the experiment with AMP-8-C\(^4\) as an additional reactant the ACP produced had the same radioactivity as the reisolated AMP, but the imidazoleglycerol phosphate produced had no detectable radioactivity. On the other hand carbon 2 of adenine was incorporated into imidazoleglycerol phosphate to the same extent as into AMP, but was not significantly incorporated into ACP.

These results indicate that the adenine ring is cleaved into two moieties: one part, containing carbon 2 of adenine, combines with ribose-5-P to form imidazoleglycerol phosphate; the remainder of the adenine ring, containing carbon 8, is converted to ACP.

Demonstration of Intermediate in Formation of ACP and Imidazoleglycerol Phosphate—A derivative of ACP was found to be an additional product when the reaction had not been permitted to proceed to completion. The derivative, which has been designated “Compound III,” is converted to ACP by hydrolysis in 0.2 N HCl at 100° for 5 minutes. It is routinely estimated by determining the increase in arylamine after acid hydrolysis. Compound III formation can also be detected spectrophotometrically since the compound has a relatively high absorption at 290 m\(\mu\). In the absence of glutamine\(^4\) there is an increased accumulation of Compound III which quantitatively corresponds to a decreased formation of imidazoleglycerol phosphate and of ACP (Table III). These observations suggest that Compound III is an intermediate in the formation of both ACP and imidazoleglycerol phosphate.

By the use of fractional precipitation with protamine it has been possible to separate the enzymes necessary for the synthesis of Compound III from those necessary for its conversion to ACP. Nucleic acids and inactive proteins were removed from extracts of E. coli, HP-1, by the addition of protamine sulfate as described in the section on “Methods.” A further addition of 4 ml of a 2% solution of protamine sulfate to 100 ml of extract resulted in the formation of a precipitate which was dissolved with 10 ml of 0.05 M reduced glutathione in 0.5 M KCl. This fraction, I, produces 10 times as much Compound III per mg of protein as does the original extract; however, it is unable to convert Compound III to ACP unless the supernatant fluid remaining after protamine precipitation, Fraction II, is added (Table IV).

Role of Reactions Leading to Imidazoleglycerol Phosphate—The essentiality of these reactions for the biosynthesis of histidine is shown by observations on several histidine auxotrophs of S. typhimurium (Table V). Extracts of strain hi F-41 are unable to produce Compound III. Extracts of strain hi A-5, although capable of producing Compound III, cannot convert this compound to ACP and imidazoleglycerol phosphate. However, extracts of strains hi A-5 and hi F-41 acting together can produce both imidazoleglycerol phosphate and ACP. These findings

supplemented with 4 \(\mu\)g of histidine per ml, unless stated other-

<table>
<thead>
<tr>
<th>Extract prepared from</th>
<th>Arylamine*</th>
<th>Imidazole†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. aerogenes, 1033 grown in minimal medium</td>
<td>0.10</td>
<td>0.07</td>
</tr>
<tr>
<td>A. aerogenes, P-14, grown in minimal medium + 10 (\mu)g/ml of guanine</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>E. coli, W grown in minimal medium</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>S. typhimurium, hi B-12 grown in minimal medium + 4 (\mu)g/ml of histidine</td>
<td>0.07</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* 5-Amino-4-imidazolcarboxamide was used as the standard.
† Imidazoleglycerol phosphate was used as the standard.
TABLE II
Formation of imidazolyglycerol phosphate and 5-amino-1-ribosyl-1-imidazolcarboxamide 5-phosphate

The reaction mixtures contained per ml: ATP, 2.1 μmoles; ribose-5-P, 4.2 μmoles; acetyl-P, 16.8 μmoles; reduced glutathione, 12.6 μmoles; glutamine, 12.6 μmoles; tri(hydroxymethyl)aminomethane, pH 8.06, 84 pmoles, MgCl₂, 16.8 μmoles; and a dialyzed protamine-treated extract of S. typhimurium, strain hi B-12, 0.33 ml (10 mg of protein per ml). Experiment 1: The total volume was 24 ml; 10.1 pmoles of ribose-5-P-1-C⁴ were added. Experiment 2: The total volume was 120 ml; 9 pmoles of AMP-8-C⁴ were added. Experiment 3: The total volume was 35 ml; 18.5 pmoles of adenine-2-C⁴ were added. In each case the reaction mixture was incubated at 37° until ACP and imidazolyglycerol phosphate formation ceased (90 minutes).

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>C⁴ compound added</th>
<th>M.A.*</th>
<th>Compound isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IGP†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>μmoles</td>
</tr>
<tr>
<td>1</td>
<td>Ribose-5-P-1-C⁴</td>
<td>6,610</td>
<td>4.9</td>
</tr>
<tr>
<td>2</td>
<td>AMP-8-C⁴</td>
<td>28,000</td>
<td>11.3</td>
</tr>
<tr>
<td>3</td>
<td>Adenine-2-C⁴</td>
<td>4,560</td>
<td></td>
</tr>
</tbody>
</table>

* M.A. (micromolar activity) = counts × minutes⁻¹ × μmoles⁻¹.
† Imidazolyglycerol phosphate.

indicate that strain hi F-41, although unable to form Compound III, can convert this intermediate to ACP and imidazolyglycerol phosphate. An extract of a histidine auxotroph which excretes imidazolyglycerol phosphate into its culture medium (strain hi B-12) contains the enzymes necessary for the synthesis of Compound III and of imidazolyglycerol phosphate and ACP. The data presented in Table V were obtained with extracts of cells which had been grown in a medium containing 4 μg of histidine per ml. If the amount of histidine in the medium is increased beyond that necessary for maximum growth (10 μg per ml) the levels of the enzymes necessary for the synthesis of Compound III, imidazolyglycerol phosphate, and ACP are reduced to 30% of the values reported in Table V. Histidine has a similar effect on the levels of these enzymes in E. coli W, an organism which does not require histidine for growth.

Inhibitory Effect of Histidine—The rate of synthesis of ACP by extracts is 50% inhibited by about 5 × 10⁻⁵ M L-histidine (Table VI). Histidine was found to act by inhibiting the formation of Compound III. This effect is illustrated in Fig. 1. The synthesis of Compound III results in an increased absorption by the reaction mixture at 290 nm. The addition of a large amount of histidine stopped further synthesis of Compound III as indi-
The observation by Broquist and Snell (17) that the purine requirement of Lactobacillus casei is reduced by the addition of histidine to the growth medium suggested that histidine might be derived, at least in part, from one of the purines. In subsequent experiments Mitoma and Snell (7) found that carbon 2 of guanine was incorporated into carbon 2 of histidine. Extension of these observations to E. coli established that the \( N_1-C_2 \) portion of histidine is derived entirely from carbon 2 of guanine and an attached nitrogen atom (6). Additional incorporation experiments carried out by Neidle and Waelsch (8) proved that it is either nitrogen 1 or nitrogen 3 of guanine which is incorporated into histidine and offered evidence that nitrogen 1 is involved. In later experiments these authors found that adenine is a more immediate precursor of histidine than is guanine, and that the number 3 nitrogen of histidine is derived from the amide nitrogen of glutamine by growing cells (18).

These experiments and the observations reported in the present paper on the synthesis of the histidine precursor, imidazoleglycerol phosphate, suggest the following pathway for the synthesis of the imidazole ring of histidine:

\[
\text{Glutamine} 
\rightarrow \text{Ribose-5-P} 
\rightarrow \text{ATP} 
\rightarrow \text{Compound III} 
\rightarrow \text{ACP} 
\rightarrow \text{Imidazoleglycerol phosphate}
\]

The essential role of these reactions in histidine biosynthesis is shown by the fact that their elimination by mutation causes a requirement for histidine for growth.

The utilization of the \( N_1-C_2 \) portion of the adenine ring of ATP for imidazoleglycerol phosphate synthesis and the concomitant production of ACP is part of a cyclic mechanism in which the purines function catalytically:

\[
\text{IMP} \rightarrow \text{AMP} \rightarrow \text{ATP} 
\rightarrow \text{Ribose-5-P} 
\rightarrow \text{ACP} 
\rightarrow \text{Imidazoleglycerol phosphate} 
\rightarrow \text{Histidine}
\]

This cyclic mechanism should be inoperative in cells growing with excess histidine, which is a potent inhibitor of the reaction that yields Compound III. This inhibitory effect has been shown to be essential for the regulation of histidine biosynthesis (19).
Furthermore, the ability of histidine to inhibit Compound III synthesis suggests a mechanism by which histidine could reduce the requirement for adenine in those organisms that are unable to convert ACP to AMP. In such organisms histidine synthesis will be accompanied by the conversion of adenine to a non-utilizable compound, ACP. The addition of histidine to the growth medium should prevent this diversion and, therefore, reduce the amount of exogenous adenine required for growth. This explanation may account for the observation (17) that histidine spares the purine requirement of L. casei and for the similar finding that histidine reduces the purine requirement of a mutant of E. coli, strain HP-1, blocked between ACP and IMP (5). As would be predicted by this scheme, histidine does not alter the purine requirements of several auxotrophs blocked in reactions other than the conversion of ACP to AMP.

Finally we wish to call attention to the effect of histidine on Compound III synthesis as still another example of the type of inhibition by an end product postulated by Umbarger and Brown (20) to underlie the feedback control of many biosynthetic processes.

SUMMARY

The synthesis of d-erythro-imidazoleglycerol phosphate ester, a precursor of histidine, has been observed in cell-free preparations from three species of enteric bacteria. Several individual enzymatic steps in the over-all reaction have been demonstrated. Each of these steps is essential for the biosynthesis of histidine.

Evidence is presented that imidazoleglycerol phosphate is produced from ribose 5-phosphate, the amide nitrogen of glutamine, and the N-1, C-2 portion of the adenine ring of adenosine triphosphate. The remainder of the adenosine triphosphate appears as 5-amino-1-ribosyl-4-imidazolecarboxamide 5-phosphate. The latter compound, an intermediate in purine biosynthesis, can be reconverted to adenosine triphosphate. Thus, a cyclic process is involved in the synthesis of the imidazole ring of histidine.

Adenosine triphosphate has a dual catalytic role in this process: it provides the energy for the reactions, and its adenine ring is the source of the N-1, C-2 portion of the imidazole ring of histidine.

REFERENCES