SYNTHESIS OF PURINE INTERMEDIATES BY A CELL-FREE EXTRACT OF ESCHERICHIA COLI

SAMUEL H. LOVE

Division of Biochemistry, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

Received for publication March 15, 1956

A purine-requiring mutant (strain W-11) of Escherichia coli strain K-12, has been shown to synthesize 5-aminoimidazole riboside precursors (Love and Gots, 1955). This strain was thought to lack the enzymes responsible for the conversion of aminoimidazole ribotide to purines.

On the basis of information on the biosynthesis of purines carried out with enzyme systems from pigeon liver (Buchanan et al., 1955), an attempt was made to synthesize the ribotide of aminoimidazole with extracts of this mutant. It was found that under proper experimental conditions these extracts carried out reactions directed toward the biosynthesis of purines or purine intermediates. The product of the reaction was, however, 5-amino-4-imidazolecarboxamide ribotide rather than 5-aminoimidazole ribotide.

MATERIALS AND METHODS

5-amino-4-imidazolecarboxamide ribotide-2-C¹⁴, which was synthesized with the aid of the pigeon-liver system from 5-amino-4-imidazolecarboxamide-2-C¹⁴ and 5-phosphoribosylpyrophosphate (PRPP), was characterized as previously described (Flaks and Buchanan, 1954) and was kindly supplied by Dr. Joel G. Flaks of this laboratory. Crystalline adenosine-5-triphosphate (ATP) was purchased from Pabst Laboratories. Ribose-5-phosphate was obtained from adenosine-5-phosphate (Khym et al., 1954), and PRPP was prepared according to a modified method of Remy et al. (1955). The other substrates were commercial products.

Preparation of cell-free extract. E. coli strain W-11 was stored on a chemically-defined medium (Gots and Chu, 1952) containing suboptimal purines for growth (6 μg per ml). A seed culture (150 ml) on the chemically defined medium was used to inoculate 10 L of media containing 6 μg per ml of hypoxanthine. Maximal growth was obtained in 12 hr by incubation at 37 C with intense aeration. The cells were harvested in a refrigerated Sharples centrifuge, washed in phosphate buffer (Schulman et al., 1952) and resuspended in the same buffer to a final concentration of 15 mg per ml dry weight. The final volume was approximately 40 ml. The suspension was then subjected to ultrasonic oscillation for 30 to 40 min, the length of time depending upon the degree of rupture of the cells as observed visually. The extract was treated in a Spinco centrifuge for 30 min at 100,000 x G. After centrifugation, the supernatant solution was dialyzed overnight against 2 L of phosphate buffer (0.01 M, pH 7.2) at 2 C. The dialyzed extract was lyophilized and stored at -20 C. The lyophilized extract did not lose activity during the first 2 weeks of storage under these conditions but it slowly lost activity after a longer period of time.

After incubation, the proteins were precipitated in the cold with a final concentration of 5 per cent (v/v) HClO₄. The precipitate was removed by centrifugation and the supernatant solution was adjusted to pH 9 with 6 N KOH. The supernatant solution was chilled to facilitate removal of as much KClO₄ as possible prior to addition of the solution to a “dowex 1 Cl⁻” column (0.8 x 8.5 cm), 200–400 mesh. The column was washed with 150 ml of water and the reaction products eluted with 0.003 N HCl.

Measurement of derivatives of 5-amino-4-imidazolecarboxamide. The undialyzed extract possessed a high amount of residual arylamine compound (5-aminoimidazole riboside or ribotide) which would interfere with the determination of 5-amino-4-imidazolecarboxamide as measured with the Bratton and Marshall (1939) test. However, 5-aminoimidazole derivatives may be
destroyed by heating in acid solution; therefore, 5-amino-4-imidazolecarboxamide and its pentose-containing derivatives were measured after decomposition of the aminoimidazole derivative as an acid- and heat-stable, non-acetylatable, diazotizable arylamine. Samples were made 1 N with respect to H₂SO₄, heated to 100 C for 1 hour and were cooled prior to diazotization. When dialyzed extracts were used, an unincubated control sample was used as blank for the colorimetric determination and contained little residual diazotizable arylamine.

RESULTS

Substrates required for synthesis of purine intermediates. The compounds which have been found necessary for synthesis of inosinic acid by the enzyme system of pigeon liver have been demonstrated to be formate, ribose-5-phosphate, 3-phosphoglyceric acid, ATP, glutamine, aspartic acid, glycine, and bicarbonate (Buchanan et al., 1955). When these substrates were incubated with extracts of E. coli strain W-11, a compound was formed which coupled with the Bratton-Marshall reagents to produce a purple chromophore. This compound was subsequently proven to be 5-amino-4-imidazolecarboxamide ribotide. When inquiry was made into the dispensability of the above substrates it was found that the dialyzed bacterial enzyme system had an absolute requirement for ATP, 3-phosphoglyceric acid, ribose-5-phosphate and glutamine. Glycine, formate, bicarbonate, and aspartic acid could stimulate the synthesis of purine intermediates but were not absolutely essential in this crude system for synthesis (table 1). In all probability, this lack of absolute requirement for these latter compounds indicates that they may be synthesized from other compounds in the incubation medium. An example of this is the recent observation by Weissbach and Horecker (1955) that in certain plant extracts glycine may be formed from ribose-5-phosphate in the presence of an appropriate source of nitrogen compound.

5-Phosphoribosylpyrophosphate may substitute for ribose-5-phosphate, but it is not known whether the former compound participates directly or through conversion to the latter. Deoxyribose-5-phosphate could not substitute for ribose-5-phosphate.

As shown in figure 1 the rate of synthesis of 5-amino-4-imidazolecarboxamide ribotide is

<table>
<thead>
<tr>
<th>Substrate</th>
<th>μmoles</th>
<th>Per Cent Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Less: Glycine</td>
<td>28</td>
<td>80</td>
</tr>
<tr>
<td>Na-formate</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>KHCO₃</td>
<td>120</td>
<td>46</td>
</tr>
<tr>
<td>Glutamine</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Adenosine-triphosphate</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Na-3-phosphoglycerate</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>Ribose-5-phosphate</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Plus: Na-aspartate</td>
<td>15</td>
<td>130</td>
</tr>
</tbody>
</table>

The dialyzed bacterial extract (1 ml) was incubated with the substrates and phosphate buffer (0.5 ml, pH 7.2) in a total of 2.2 ml, at 37 C, for 1 hour. The per cent activity was a measure of the chromophore produced with the Bratton and Marshall reagents, as described under Methods. The maximum synthesis obtained in the complete system was usually 30 to 40 μg per ml.

Figure 1. Synthesis of 5-amino-4-imidazolecarboxamide ribotide. The incubation mixture was the same as the complete system plus aspartic acid as described in table 1. Aliquots of 0.25 ml were removed, made up to 2 ml, and tested as described under Methods. 540 mμ KU-BM per ml represents the absorption at 540 mμ in a Klett-Summerson colorimeter of the chromophore produced with the Bratton and Marshall reagents. greatest during the first hour of reaction and slows down considerably thereafter. Apparently the concentration of ATP is an important factor in the synthesis. The optimal concentration of
concentrations above ATP is respectively. Marshall, with diazotizable and BM+ conditions experimental product amino-4-imidazolecarboxamide ribotide-8-C14. The sodium acetate, and removed by centrifugation. Upon the addition of 3 to 4 vol of acetone the barium salt of the ribotide was precipitated. This salt, which was converted to the sodium form by metathesis with sodium sulfate, was shown to contain 80 per cent of both the diazotizable arylamine and the radioactivity. The sodium salt was made alkaline (pH 9) and ion exchange chromatography carried out on a column of Dowex 1 Cl as described in a previous section. The elution of the compounds was followed by measurement of radioactivity, ultraviolet absorption and by reaction with the Bratton-Marshall reagent. The elution of the arylamine as tested colorimetrically coincided closely with the elution of the radioactive material (figure 3). Since the specific activity of the recovered arylamine was approximately 100 cpm per µmole, it may be assumed that the authentic sample of radioactive carboxamide ribotide and the product of the reaction in bacterial extracts are eluted at identical positions on the Dowex 1 Cl columns and are therefore presumably the same compound.

Further evidence for the identity of the reaction product of the bacterial extract as 5-amino-4-imidazolcarboxamide ribotide has come from a comparison of this compound with an authentic sample of the carboxamide ribotide by the method of paper chromatography. Both compounds had an Rf of 0.05 in a solvent system composed of propyl alcohol: 0.2 × NH₄OH (3:1). Under these conditions the carboxamide riboside and the free carboxamide have Rf values of 0.40 and 0.55, respectively.

**DISCUSSION**

An interesting result of the experiments designed to achieve synthesis of purine intermediates in cell-free systems of *Escherichia coli*

**Figure 2.** Effect of adenosine triphosphate concentration on synthesis of 5-amino-4-imidazo-1carboxamide ribotide. Incubation mixtures were the same as shown in figure 1 but with varying concentrations of ATP. The incubation time was 1 hour. 100 per cent = approximately 30 µg 5-amino-4-imidazo-carboxamide ribotide per ml.

**Figure 3.** Ionexchange chromatography of reaction product with an authentic sample of 5-amino-4-imidazo-carboxamide ribotide-8-C14. Experimental conditions described under Results. BM+ and cpm represent the fractions which are diazotizable with the method of Bratton and Marshall, and those which are radioactive, respectively.

**Identification** of 5-amino-4-imidazo-carboxamide ribotide as a product of reaction. In an experiment with 36 ml of reaction mixture, 16 µmoles of a derivative of 5-amino-4-imidazo-carboxamide were synthesized as measured by diazotization. The reaction mixture was deproteinized with HClO₄ and the supernatant solution retained (see Materials and Methods). Then 1.1 µmoles of 5-amino-4-imidazo-carboxamide ribotide-2-C14 with a specific activity of 2,500 cpm per µmole were added to the supernatant solution. Barium phosphate was precipitated upon addition of excess barium acetate and removed by centrifugation. Upon the addition of 3 to 4 vol of acetone the barium salt of the ribotide was precipitated. This salt, which was converted to the sodium form by metathesis with sodium sulfate, was shown to contain 80 per cent of both the diazotizable arylamine and the radioactivity. The sodium salt was made alkaline (pH 9) and ion exchange chromatography carried out on a column of Dowex 1 Cl as described in a previous section. The elution of the compounds was followed by measurement of radioactivity, ultraviolet absorption and by reaction with the Bratton-Marshall reagent. The elution of the arylamine as tested colorimetrically coincided closely with the elution of the radioactive material (figure 3). Since the specific activity of the recovered arylamine was approximately 100 cpm per µmole, it may be assumed that the authentic sample of radioactive carboxamide ribotide and the product of the reaction in bacterial extracts are eluted at identical positions on the Dowex 1 Cl columns and are therefore presumably the same compound.

Further evidence for the identity of the reaction product of the bacterial extract as 5-amino-4-imidazo-carboxamide ribotide has come from a comparison of this compound with an authentic sample of the carboxamide ribotide by the method of paper chromatography. Both compounds had an Rf of 0.05 in a solvent system composed of propyl alcohol: 0.2 × NH₄OH (3:1). Under these conditions the carboxamide riboside and the free carboxamide have Rf values of 0.40 and 0.55, respectively.

**DISCUSSION**

An interesting result of the experiments designed to achieve synthesis of purine intermediates in cell-free systems of *Escherichia coli*
strain W-11 has been the fact that 5-amino-4-imidazolcarboxamide ribotide was the chief product of reaction instead of 5-aminoimidazole ribotide. Preliminary experiments indicate that complete purine compounds also are probably formed de novo by the enzyme systems of this bacterial extract. It is therefore evident that the requirement for purines by this mutant strain is not due to a lack of enzymes involved in the synthesis of purine compounds and that the accumulation of 5-aminimidazole riboside by the whole cells of this strain must be caused by other factors at present not understood. A system employing an adenine-requiring yeast was shown to be capable of synthesizing purines but in quantities insufficient to meet the requirements of the whole cells (Abrams, 1952).

SUMMARY

Cell-free extracts of Escherichia coli strain W-11 have been prepared which can synthesize purine intermediates from elementary precursors. Glutamine, ribose-5-phosphate, adenosine triphosphate and phosphoglyceric acid were necessary substrates for this biosynthetic reaction. The main product of the reaction was 5-amino-4-imidazolcarboxamide ribotide. This was in contrast to the results obtained with whole cells which mainly produced 5-aminimidazole riboside. It had been believed previously that strain W-11 possessed a genetic block which prevented the conversion of 5-aminimidazole compounds to 5-amino-4-imidazolcarboxamide ribotide and to purines. The experiments with extracts show, however, this metabolic block was not caused by a lack of enzymes required for the further metabolism of 5-aminimidazole compounds.

REFERENCES


