PURINE METABOLISM IN BACTERIA

II. FACTORS INFLUENCING BIOSYNTHESIS OF 4-AMINO-5-IMIDAZOLECARBOXAMIDE BY ESCHERICHIA COLI*

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An important link in the chain of reactions leading to the biosynthesis of purines was found with the recognition (1), isolation (2), and identification (3) of 4-amino-5-imidazolecarboxamide (AICA).

By means of isotopic tracers, AICA was shown to be incorporated into purine components by pigeon liver homogenates (4), yeast (5), and intact mammals (6, 7). This apparently occurs via a pentose form, presumably the ribotide, to yield inosinic acid (8-10).

In nature, AICA has been found only as an accumulated product in cultures of gram-negative bacilli, primarily Escherichia coli. Such accumulation has been obtained by the use of inhibitory analogues of p-aminobenzoic acid (1, 11, 12) and folic acid (11, 13), or by genetic blocks involving defects in purine synthesis (14) and p-aminobenzoic acid synthesis (11). In all of these cases, the accumulation of AICA can be related to a prevention of its utilization by interference with the single carbon addition required for ring closure at the 2 position of the purine nucleus. However, Stewart and Sevag (15) reported that non-inhibited E. coli cultures accumulated AICA in a casein hydrolysate-glucose medium.

With an aim toward determining the factors required for purine synthesis, a number of workers have examined the effects of various environmental manipulations on the production of AICA by growing E. coli cultures (10-12, 15-19). The use of growing systems for this type of analysis immediately limits the extent of the factors that can be examined. Any agent which alters the growth response will, a priori, affect the accumulation. The components of the medium, however simple, must be present, and postulated metabolic derivatives of these components cannot be adequately assayed unless they can substitute for growth requirement. In order to circumvent these difficulties and with the eventual goal of resolving purine synthesis to an enzymatic level, we have examined the production of AICA by non-proliferating cell suspensions of E. coli. This was made possible by the use of a purine-requiring mutant which is genetically

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impaired in the conversion of AICA or its ribose derivative, and hence accumulates AICA. In environments lacking a purine, the bacteria remain quiescent with respect to growth and cell multiplication. Using these conditions as an index of purine-synthesizing potentialities, we have examined some factors which control the synthesis of AICA.

Materials and Methods

The organism used was E. coli, strain B-96, a purine-requiring mutant of strain B. Growth requirements and the accumulation of AICA by this mutant have been described (14).

Bacterial suspensions were prepared from an overnight culture in tryptose broth containing glucose (0.2 per cent). The cells were washed twice by centrifugation and resuspended in physiological saline. The concentration of the suspension was adjusted on a basis of mg. of dry weight per ml. by means of previously calibrated turbidity standards. The test systems were routinely carried out in the presence of 1/15 phosphate buffer at pH 7.2 in a water bath at 37°. The components of the reacting systems varied for the particular experiment and will be described where indicated. At prescribed intervals, the reactions were terminated with trichloroacetic acid. The product was centrifuged, and the clear supernatant solutions were used for analysis.

AICA was routinely determined by measurement as a diazotizable amine by the Bratton-Marshall method (20). This test does not permit distinction between the base and the ribose derivatives of AICA, which are also known to accumulate (21). Separate analysis for the riboside and ribotide forms of AICA was not attempted in these studies, and hence the figures represent total AICA in all forms.

Glucose was measured as a reducing sugar by the Folin-Wu method (22). Pyruvate was measured by the method of Bueding and Wortis (23). The orcinol method described by Drury (24) was used to measure pentoses.

Results

Active synthesis of AICA, progressing linearly with time, was obtained with the washed whole cell suspensions in the presence of glucose and an inorganic ammonium salt. Sonically prepared cell-free extracts and acetone-dried and vacuum-dried cells were inactive. Varying amounts of glucose gave a typical substrate saturation curve with optimal concentration at 0.02 M. In the absence of a nitrogen source, glucose alone was ineffective. Though the nitrogen requirement could be satisfied by simple inorganic salts, such as ammonium chloride or ammonium sulfate, casein hydrolysate, at an optimal concentration of 0.1 per cent, was several times more efficient in both rate of synthesis and maximal yield. This can be
seen in Fig. 1. The rate of synthesis with casein hydrolysate consistently ranged between 0.3 and 0.4 \( \gamma \) of AICA per minute per mg., as compared with 0.06 to 0.08 \( \gamma \) with ammonium salts. In the absence of glucose, casein hydrolysate alone or in the presence of an ammonium salt had no effect. A requirement for glucose in the accumulation of AICA by sulfonamide-inhibited \( E. coli \) cultures has been reported by Stetten and Fox (2) and Sevag and Stewart (19).

That AICA synthesis is a direct consequence of glucose metabolism was shown by a concomitant measurement of glucose utilization with AICA formation. Fig. 2 shows that, after a 15 minute lag, the rate of AICA

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**Fig. 1**

Rate of formation of 4-amino-5-imidazolecarboxamide as determined by source of nitrogen. The system consisted of \( E. coli \) cells (0.5 mg. per ml.), glucose (0.02 M), casein hydrolysate (CH, 0.1 per cent) or \( \text{NH}_4\text{Cl} \) (0.1 per cent), in \( \text{M}/15 \) phosphate buffer at pH 7.2. Mixtures incubated at 37\( ^\circ \), and aliquots removed at the indicated times. The reaction was terminated with trichloroacetic acid.

**Fig. 2**

Rate of formation of AICA as compared with rate of glucose utilization. The system contained 0.1 per cent casein hydrolysate. All other components and treatment as indicated in Fig. 1. “AICA reversed” was calculated by subtracting the per cent of AICA formed from 100.
production is directly parallel to the rate of glucose disappearance. When glucose could no longer be detected as a reducing sugar, AICA accumulation ceased. This relationship served to explain the paradoxical results obtained when AICA synthesis was measured as a function of cell concentration. As can be seen in Fig. 3, though the rate of synthesis is directly proportional to cell mass, up to a maximum, the eventual amount produced decreased with increased concentration of cells. Since AICA production

![Graph showing yield and rate of formation of AICA as a function of cell concentration](http://www.jbc.org/)  
**Fig. 3.** Maximal yield and rate of formation of AICA as a function of cell concentration. The components and conditions were as indicated in Fig. 2.

depends on the availability of glucose, it would be expected that an increase in cell concentration would allow a more rapid exhaustion of the carbon and energy sources provided by the glucose, thus leading to a rapid initial rate and early termination of AICA production.

Apparently, glucose can serve as an oxidizable substrate for the provision of the energy required for synthesis as well as a source of the carbon units which function as building blocks. Stewart and Sevag (15) tested a variety of carbon sources and found that L-arabinose, particularly in the presence of pyruvate, had a marked effect on the accumulation of AICA in growing systems. Our results with these and several other substrates were, in part, comparable to their findings. A wide variety of possible
carbon sources were tested for their ability to replace glucose in the presence of casein hydrolysate. The amount of AICA formed in 3 hours by 0.5 mg. of cells per ml. with 0.02 M of glucose was equated as 100 per cent activity. With equimolar quantities, the following substances showed activity as indicated: potassium gluconate, 154; D-galactose, 103; glucose-1-phosphate, 66; L-arabinose, 64; uridine, 22; and glucose-6-phosphate, 11. Thus, as a carbon and energy source for the synthesis of AICA, gluconate is more efficient than glucose.

Since the phosphate esters of glucose may be under the restriction of permeability barriers, glucose-6-phosphate was also tested with a cell-free extract prepared by sonic vibration, but no activity could be demonstrated. Since uracil was inactive, the activity with uridine may be due to a liberation of ribose-1-phosphate. L-Arabinose, which has been shown to be more efficiently utilized than glucose for assimilatory purposes in E. coli (25), will be discussed later as a special case involving adaptive processes.

As a substitute for glucose, no measurable activity could be found with D-arabinose, D-ribose, L-xylose, pyruvate, succinate, fumarate, lactate, glycerol, phosphoglyceric acid, β glycerophosphate, bicarbonate, acetyl phosphate, choline, betaine, formate, acetate, citrate, α-ketoglutarate, malate, and ascorbic acid. When these substances were tested in the presence of a suboptimal supply of glucose (0.005 M), the last six listed were found to be inhibitory, ranging from 30 per cent inhibition with citrate to 60 per cent with malate and ascorbic acid. Pyruvate and fumarate showed a slight stimulation (10 to 20 per cent) of the reduced glucose activity.

Effects similar to these were obtained when L-arabinose was used as the substrate, except that ascorbic acid was completely inhibitory and pyruvate and fumarate were more stimulatory in that their presence permitted a 2- to 3-fold increase in the yield of AICA. Stewart and Sevag (15) had previously noted an increased accumulation of AICA with a combination of L-arabinose and pyruvate. The ability of pyruvate to stimulate AICA production from L-arabinose was evident only during the first 3 to 4 hours of incubation. After this, synthesis progressed rapidly with L-arabinose alone until it was equal to the level obtained earlier with the addition of pyruvate. This delayed response with L-arabinose alone is in accord with the observations by Siegel and Clifton (25) that the enzymes involved in L-arabinose utilization are adaptive. It is difficult to understand why pyruvate, which by itself is bland, should nullify the delayed synthesis obtained with L-arabinose. That pyruvate did not enhance L-arabinose utilization was shown by the finding that no utilization of the pentose occurred until the pyruvate had been entirely consumed.
When the cell suspensions were prepared with bacteria previously grown in the presence of L-arabinose, the lag in the synthesis of AICA no longer occurred with L-arabinose, nor did pyruvate show any stimulatory action. The activity with glucose and stimulation by fumarate were unaltered by previous growth in L-arabinose. With the arabinose-grown cells the rate of synthesis of AICA did not parallel the rate of L-arabinose utilization, as was the case with glucose (see Fig. 2), but exceeded it by a factor of 2. That is, AICA was synthesized twice as fast as L-arabinose was consumed.

Fig. 4. The effect of aeration on the rate of formation of AICA. System as described for Fig. 2. "Aerobic" curve obtained by bubbling air through the reaction mixture and "Anaerobic" by similar treatment with pure nitrogen.

On an economic basis, however, L-arabinose was superior to glucose in that the economic coefficient (moles consumed per mole of AICA formed) was 50 to 60 for L-arabinose compared with 110 for glucose.

Under aerobic conditions both the rate of formation and the total yield were increased 2 to 3 times over that obtained anaerobically. This was verified first with the use of Thunberg tubes from which the air had been removed and replaced with nitrogen. Both series of tubes were mechanically shaken during the reaction. Measurements with time were facilitated by bubbling nitrogen through one reaction mixture and air through another at the same rate. The results of such an experiment are depicted in Fig. 4. It can be seen that a considerable enhancement of AICA formation occurred under the aerobic condition.
The effect of casein hydrolysate was investigated by analyzing the influence of synthetic amino acid mixtures on the production of AICA. This was done by single and multiple additions and depletions in both the presence and absence of inorganic nitrogen with glucose as the carbon and energy source. The amino acids were tested in a final concentration of 100 γ per ml of the L form. Without any other nitrogen source, significant synthesis was demonstrated with serine, glutamic acid, or aspartic acid. A mixture of these three amino acids could substitute completely

for ammonium salts. Except for a slight stimulation with aspartic acid (Table I), these amino acids did not affect the activity obtained with ammonium chloride. This would indicate their non-specific role as an alternative source of ammonia via the amino acid-deaminating mechanisms known to be possessed by this organism. More significance could be placed upon those amino acids which were able to increase the yield obtained with inorganic nitrogen. As is shown in Table I, these included, in the order of effectiveness, histidine, threonine, glycine, and aspartic acid. When cysteine, which alone was inactive, was added to a mixture of these four stimulatory amino acids, 80 per cent of the total casein hydrolysate yield was obtained. In the absence of ammonium chloride, further addition of serine and glutamic acid was required to maintain this

TABLE I

Effect of Amino Acids on AICA Synthesis

<table>
<thead>
<tr>
<th>Supplement</th>
<th>AICA γ per ml</th>
<th>Per cent effect over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.8</td>
<td>(Control 0)</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>16.7</td>
<td>+146</td>
</tr>
<tr>
<td>Histidine</td>
<td>11.3</td>
<td>+68</td>
</tr>
<tr>
<td>Threonine</td>
<td>10.3</td>
<td>+53</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.8</td>
<td>+30</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8.1</td>
<td>+20</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.7</td>
<td>+13</td>
</tr>
<tr>
<td>Cysteine</td>
<td>5.7</td>
<td>-15</td>
</tr>
<tr>
<td>Homoserine</td>
<td>3.6</td>
<td>-47</td>
</tr>
<tr>
<td>Valine</td>
<td>2.5</td>
<td>-48</td>
</tr>
<tr>
<td>α-Aminobutyric acid</td>
<td>2.7</td>
<td>-61</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.6</td>
<td>-62</td>
</tr>
</tbody>
</table>

The system consisted of cells (0.5 mg per ml.), glucose (0.02 M), and NH₄Cl (0.1 per cent), in m/15 phosphate buffer at pH 7.2. Amino acids added to final concentration of 100 γ per ml. Mixtures were incubated at 37° for 3 hours and terminated with trichloroacetic acid. The following amino acids were within ±10 per cent of the control and considered to have no significant action: lysine, isoleucine, leucine, tyrosine, phenylalanine, proline, glutamic acid, serine, alanine, and homocystine.
activity. Table I also shows the inhibitory qualities of methionine, α-aminobutyric acid, valine, and homoserine.

Negative results were obtained with a series of imidazole and malonic acid derivatives which, on a structural basis, might conceivably have served as precursors to AICA. These included imidazole, imidazole-4,5-dicarboxylic acid, imidazole-4-carboxamide,1 4-amino-5-imidazolecarboxamide,2 4-formamido-5-imidazolecarboxamide (FAICA),3 aminomalonic acid,4 malonamidine,3 aminomalonamidine,3 aminomalonamidine (AMAA),3 and formamidoaminomalonamidine (FAMAA).3 Some of the malonic acid derivatives have also been shown to be unable to serve as purine precursors in pigeon liver systems (26). AICA was increased with the two formamido derivatives, but this occurred even in the absence of cells and could be related to spontaneous hydrolysis of the formyl group in the case of FAICA or spontaneous cyclization in the case of FAMAA (27). A substance which was chromogenic with the Bratton-Marshall reagents, but which had an ultraviolet spectrum different from that of AICA, was found on incubation of the cells in the presence of glucose and AMAA (AICA lacking the ureide carbon of the imidazole ring). Though no such substance was formed with glucose in the absence of cells, similar substances could be obtained spontaneously by merely mixing AMAA with carbonyl-containing compounds such as pyruvic acid, α-ketoglutaric acid, or formaldehyde.4 In the presence of the cells, glucose apparently was metabolized to a keto compound which could react spontaneously with AMAA, yielding a diazotizable amine. It is of interest to note that this did not occur when L-arabinose was utilized.

**DISCUSSION**

The carbon and energy requirements for the synthesis of AICA by the *E. coli* mutant described here were efficiently supplied by glucose. L-Arabinose was also an efficient carbohydrate for this purpose, and its activity is in accord with the observations of Siegel and Clifton (25) that this pentose can be utilized efficiently as a source of both the carbon and energy for the synthetic processes of *E. coli*. The efficiency of gluconate in replacing glucose and the stimulation by aeration suggest that an important pathway for AICA synthesis involves the aerobic gluconate pathway of glucose dissimilation. Since AICA occurs primarily as a riboside

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1 Obtained through the courtesy of Dr. Karl Päster of Merck and Company, Inc.
2 Obtained through the courtesy of Dr. M. Earl Balis of the Sloan-Kettering Institute for Cancer Research, New York.
3 Obtained through the courtesy of Dr. Walter Brooks and Dr. John M. Buchanan of the Department of Physiological Chemistry, University of Pennsylvania.
4 This reaction was first recognized and called to our attention by Dr. J. M. Buchanan (personal communication).
(or ribotide), a greater dependence on the gluconate pathway would be anticipated because it is this pathway which has been indicated as the source of ribonucleotides in \textit{E. coli} \cite{28}.

Both Stetten and Fox \cite{2} and Sevag and Stewart \cite{19} found that aeration of a sulfonamide-inhibited culture of \textit{E. coli} prevented the accumulation of AICA. Our findings that aeration accelerated AICA formation may be an expression of an inherent difference in the quality of the two systems, the nature of which remains unknown.

The stimulatory action of glycine is in accord with the well established rôle of this amino acid as a purine precursor \cite{29}. Greenberg \cite{10} has shown that glycine can also be incorporated isotopically into AICA. On a theoretical calculation that approximately one-third of the AICA molecule can originate from glycine, the activity with glycine, as found here, was not as great as might have been anticipated. Indeed, on both molar and kinetic bases, threonine was found to be consistently more efficient than glycine. Ravel et al. \cite{18} reported that threonine could enhance AICA accumulation in the sulfonamide-inhibited cultures, but that it was far less efficient than glycine. They attributed this to the fact that threonine could serve as a precursor to glycine. This interpretation has been substantiated by the isotopic studies of Krasna et al. \cite{30}, but not by nutritional studies with \textit{glycineless} and \textit{threonineless} mutants of \textit{E. coli}.

The greater efficiency of threonine over glycine would indicate that its function may be more than merely a source of glycine. However, it is also possible that an endogenous supply of glycine, as supplied by \textit{de novo} synthesis or conversion from threonine, would be more efficient than an exogenous supply, since the latter may be lost by a side reaction such as oxidation.

Histidine was by far the most efficient amino acid in the enhancement of AICA synthesis. Since the imidazole nuclei of histidine and purines have an independent origin \cite{31}, it is unlikely that histidine serves as a direct precursor of AICA. A probable explanation for the marked histidine effect might be formulated along the lines of a sparing action. Thus if a common substance (\textit{e.g.}, a common carbon unit for the ureide carbon of the imidazole rings) is required for both histidine and AICA formation, then supplementation with preformed histidine would make this substance more available for AICA synthesis. Such a substance might also be in the form of a pentose intermediate serving as a 5-carbon donor for both histidine \cite{32} and the pentose derivatives of AICA.

On the basis of its depressive action on the accumulation of AICA by \textit{E. coli} cultures, methionine has been incriminated as a source of the carbon unit required for cyclization of AICA to yield purines \cite{11, 12, 17}. Since

\footnote{Unpublished observations.}
this could not be the case in the non-proliferating system studied here, which is also inhibited by methionine, it would be more likely that methionine suppresses accumulation by means of a direct inhibitory effect on the de novo synthesis of AICA. Similar mechanisms may be operating in the other inhibitory effects found. The mechanisms of these inhibitions remain obscure, but their demonstration serves to emphasize that the relief of an accumulated intermediate does not necessarily imply that the substance responsible for the relief is facilitating conversion of the product. It may be merely preventing its synthesis.

SUMMARY

By employing a purine-requiring mutant of Escherichia coli, the biosynthesis of 4-amino-5-imidazolecarboxamide (AICA) by non-proliferating cell suspensions has been described. The carbon and energy requirements for this synthesis were best supplied by glucose, gluconate, L-arabinose, D-galactose, or glucose-1-phosphate. The rate of formation was directly parallel with the rate of glucose utilization; aeration enhanced formation. The utilization of L-arabinose for AICA synthesis was adaptive. Nitrogen requirements could be supplied by inorganic ammonium salts, but more efficiently by casein hydrolysate. Serine, glutamic acid, and aspartic acid served as nitrogen donors; histidine, threonine, glycine, and aspartic acid were able to stimulate synthesis in the presence of an optimal supply of inorganic nitrogen. Methionine, a-aminobutyric acid, valine, and homoserine were inhibitory. Varying degrees of inhibition were also obtained with formate, acetate, citrate, a-ketoglutarate, malate, and ascorbic acid. A series of imidazole and malonic acid derivatives was unable to contribute to the formation of AICA.

BIBLIOGRAPHY

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