STUDIES ON THE SYNTHESIS OF 5-AMINO-4-IMIDAZOLECARBOXAMIDE RIBOTIDE BY CELL-FREE EXTRACTS OF *ESCHERICHIA COLI*

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SUMMARY

The synthesis of 5-amino-4-imidazolecarboxamide ribotide by extracts of *E. coli* was mediated through an acid-labile compound with the properties of "compound III" as described by MOYED AND MAGASANIK[3] in their work on the role of purines in histidine synthesis. Attempts to demonstrate the *de novo* route of purine synthesis were unsuccessful.

Undialyzed bacterial extracts contained hypoxanthine and adenine. The addition of these purines to dialyzed extracts inhibited the synthesis of 5-amino-4-imidazolecarboxamide ribotide, which was an index of histidine synthesis. In the presence of ATP and R-5-P, the extracts converted hypoxanthine and adenine to their respective ribotides. These studies were related to the feed-back inhibition of purine synthesis by bacterial whole cells and to histidine synthesis by the extracts.

INTRODUCTION

A cell-free extract of a purine-requiring mutant of *Escherichia coli*, strain W-11, was shown by LOVE[1] to be capable of synthesizing 5-amino-4-imidazolecarboxamide ribotide (AICAR). The significance of these studies was thought to be associated with the *de novo* synthesis of purines. Subsequent experiments were conducted to compare the bacterial system with that of pigeon liver, which had been studied so extensively[2].

Attempts to demonstrate the *de novo* route of purine synthesis were based on the conversion of [1-^{14}C] glycine, glycinamide ribotide, and formylglycinamide ribotide to AICAR by the bacterial extract. Only negative results were obtained. The bacterial system remained to be clarified by MOYED AND MAGASANIK[3,4], who demonstrated that the AICAR was not an indicator of *de novo* purine synthesis, but that it was derived from AMP in the synthesis of an amino acid, histidine. Their studies indicated that AMP reacted with PRPP in the absence of glutamine to form a "compound III"

Abbreviations: AICAR, 5-amino-4-imidazolecarboxamide ribotide; PRPP, phosphoribosylpyrophosphate; R-5-P, ribose-5'-phosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; PGA, 3-phosphoglyceric acid; IMP, inosine monophosphate; Hx, hypoxanthine; Ad, adenine; PP, pyrophosphate.

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which gave rise to AICAR upon mild acid hydrolysis. In the presence of glutamine, compound III was converted to AICAR and imidazoleglycerol phosphate, which was a precursor of histidine.

Purines were known to inhibit purine synthesis by bacterial cells[5-7]. A more thorough study of the "feed-back inhibition" of purine synthesis by bacteria was made by GOTS[8], who postulated that the mechanism may involve a shunting of the PRPP from the *de novo* route of purine synthesis. The same type of mechanism was thought to be operative in the inhibition of inosinic acid synthesis by the pigeon-liver system, as revealed by WYNGAARDEN[9].

These studies support the findings of MOYED AND MAGASANIK in addition to providing additional information relative to the "feed-back inhibition" mechanism.

MATERIALS AND METHODS

Ribose-5'-phosphate was prepared from AMP by the method of KHYM *et al.*[10]. Other chemicals were commercial products. Whatman No. 1 paper was used for the paper chromatograms, which in turn were developed with a mixture of *n*-butanol saturated with water (100 ml) and NH_4OH (15 *N*, 1 ml). Compounds capable of absorbing u.v. light were detected on the chromatograms with the aid of a Mineralight lamp.

Ion-exchange procedures employed the chloride form of Dowex-1. The eluting fluids were those described by COHN[11] for the ion-exchange separation of nucleotides.

The chromophore produced by the reaction of 5-amino-4-imidazolecarboxamide ribotide with the BRATTON-MARSHALL[12] reagents was measured with the aid of a Klett-Summerson colorimeter at 540 mμ. The final reaction volume was 5.2 ml. Pentoses were measured by the method of MEJBAUM[13], using AMP as standard. U.v. absorption spectra were determined with the aid of a Beckman Model DU spectrophotometer.

Escherichia coli, strain W-11, grown in synthetic medium containing 14 μg/ml of hypoxanthine[14] gave the highest yield of cells whose extract was active in the synthesis of AICAR. The extracts were prepared and stored as previously described[1]. The addition of reduced glutathione (10 μmoles/ml) to the cell-free extract prior to its lyophilization prolonged the enzyme activity during storage. In some cases, which were not investigated further, the activity appeared to increase following the storage

TABLE I

COMPLETE MEDIUM FOR AICAR SYNTHESIS

Substrate	*μmoles/ml*	*ml*	*Total μmoles*
R-5-P	100	0.10	10.0
Glutamine	150	0.05	7.5
Na-ATP	100	0.02	2.0
PGA	150	0.10	15.0
Glycine	150	0.10	15.0
Na-formate	300	0.10	30.0
Na-aspartate	150	0.10	7.5
$NaHCO_3$	600	0.10	60.0
Na-phosphate buffer	100	0.20	20.0
$MgCl_2$	500	0.02	100.0

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procedure. The complete substrate mixture for the synthesis of AICAR by the bacterial extract included all of the substrates as originally described by LOVE[1]. The concentrations have been modified and are listed in Table I. The minimal medium for AICAR synthesis consisted of the first four substrates listed, plus the $MgCl_2$ and phosphate buffer.

The optimal concentration of cell-free extract required for the synthesis of AICAR was determined after each extract preparation, or after extended storage of the extract. This procedure was necessary to insure comparable results from one extract preparation to the next. The optimal concentration of extract was approximately 6.4 mg/ml, as illustrated in Fig. 1. If the maximum synthesis of AICAR by an extract did not exceed 600 Klett units/ml fresh extracts were prepared.

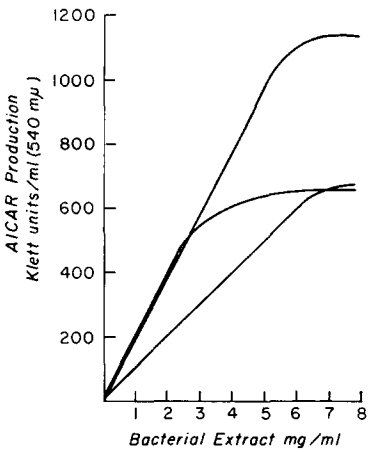

Fig. 1. Optimal bacterial extract concentration for AICAR production. Varying concentrations of lyophilized *E. coli* extract were incubated with the complete medium, listed in Table I (final volume = 1.24 ml) for 1 h at 37°. AICAR was measured by the method of BRATTON AND MARSHALL.

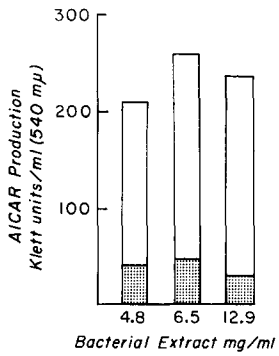

Fig. 2. Detection of Compound III production. The various concentrations of *E. coli* extract were incubated with the minimal medium described under METHODS, in a total volume of 1.24 ml, for 25 min at 37°. The shaded areas represent the amount of AICAR produced by the extracts in the absence of glutamine and without hydrolysis. The clear areas represent the amount of AICAR produced by hydrolysis of compound III in 2.5% HCl at 100° for 20 min.

RESULTS

Confirmation of the route of AICAR synthesis

Detection of compound III, as described by MOYED AND MAGASANIK[3], was facilitated by the case with which it can be hydrolysed under mild acid conditions. The cell-free extract of *E. coli*, strain W-11, was examined indirectly for its ability to synthesize this histidine intermediate by measuring before and after hydrolysis the amount of AICAR produced in the incubation mixture. Fig. 2 illustrates the 5-fold increase in diazotizable amine following acid hydrolysis. All procedures, following incubation, were conducted in an ice bath owing to the lability of compound III. The increase in AICAR after hydrolysis indicated that compound III was synthesized by the *E. coli* extract. Subsequent studies were interpreted in the light of this new information; *i.e.*, the AICAR resulted not as a consequence of *de novo* synthesis but was a by-product in the synthesis of histidine.

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Identification of hypoxanthine and adenine in the bacterial extract

Dialyzed extracts were noted to be more active than undialyzed extracts in the synthesis of AICAR. The dialysate contained u.v.-absorbing substances, and this prompted us to examine the bacterial extracts directly for their presence.

Extracts were prepared as previously described up to the procedure of dialysis. Samples of the extract were taken before and after dialysis and were deproteinized with $HClO_4$ (5 % final concn.), neutralized with KOH and chilled. Aliquots of the clear supernatant solutions were spotted on Whatman No. 1 paper for ascending chromatography, and were developed as described under METHODS. After 16 h of migration, 2 u.v.-absorbing compounds that were not detected in the dialyzed extract were obtained from the undialyzed bacterial extract. The 2 unknown compounds had R_F values identical to those of authentic samples of hypoxanthine (0.13) and adenine (0.36). Individual samples were eluted with 0.1 *N* HCl at 37° for 3 h. The u.v. absorption spectra of the unknown compounds confirmed their identity as corresponding to hypoxanthine and adenine.

The inhibition of AICAR synthesis by hypoxanthine and adenine

The dialyzed bacterial extract was examined for its ability to synthesize AICAR in the presence of varying concentrations of adenine and of hypoxanthine. Both compounds were found to be inhibitory for AICAR synthesis as illustrated in Fig. 3. Maximum inhibition was obtained with approximately 0.6 μmole/ml of each purine. The effects of other purines or purine derivatives were not examined.

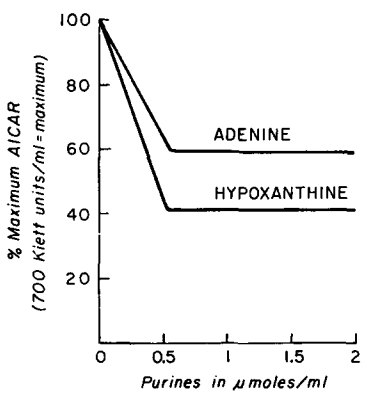

Fig. 3. The inhibition of AICAR production by adenine and hypoxanthine. Individual systems consisted of the complete medium listed in Table I plus 8 mg of *E. coli* extract. Adenine or hypoxanthine were added, as indicated, to the different systems, which were made up to 1.24 ml with water. Incubation was for 1 h at 37°. AICAR was measured by the BRATTON AND MARSHALL method.

Fate of hypoxanthine and adenine in the bacterial extract

Purine bases were known to be converted to their respective ribotides when they were incubated with PRPP in the pigeon-liver system[15]. The same reactions were thought to be operating in the bacterial system since the necessary substrates were present. The synthesis of PRPP had been demonstrated with extracts of *E. coli*[16]. The reactions were as follows:

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1. ATP + R-5-P ⟶ AMP + PRPP
2. Hx + PRPP ⟶ IMP + PP
   Ad + PRPP ⟶ AMP + PP

To test for these reactions, the bacterial extracts were incubated with the purine in question with a modified minimal medium required for the synthesis of AICAR.

The experiments which employed hypoxanthine consisted of ATP (20 μmoles). R-5-P (100 μmoles), PGA (150 μmoles), $MgCl_2$ (100 μmoles), sodium phosphate buffer, pH 8 (400 μmoles), hypoxanthine (5.64 μmoles), and lyophilized, dialyzed bacterial extract (64.5 mg) in a total of 12.4 ml. As soon as the bacterial extract was mixed with the substrates, 6 ml of the incubation mixture were removed for a control and added to $HClO_4$ (5 % final concn.) in an ice bath to stop the reaction. The protein was removed following centrifugation. The supernatant solution was adjusted to pH 8 with KOH, rechilled, and the insoluble $KClO_4$ was discarded after centrifugation.

The remaining reaction mixture used as a test system was incubated at 37° for 1 h. After incubation, the test system was treated in the same way as the control system.

The final supernatant solutions from the control and test systems (4.5 ml) were adsorbed on separate Dowex-1 columns (0.8·7.5 cm) in the chloride form. The method of elution was essentially that of COHN[11]. The u.v.-absorbing compounds were identified by their relative elution pattern, pentose content, and their u.v. absorption spectra.

To determine whether or not the bacterial extract was able to convert adenine to AMP, the medium described above was employed, but adenine (10 μmoles) was substituted for hypoxanthine and was added to one-half of the incubation mixture. Instead of taking an aliquot at zero time to serve as a control, it was necessary to incubate half of the incubation mixture without adenine and half of the incubation mixture with adenine. Adenine (10 μmoles) was added to the control after incubation. The control and test systems were treated as described for the experiment containing hypoxanthine. This type of control was necessary since AMP was formed from ATP in the absence of added adenine, as demonstrated in Table II. The test for AMP formation from adenine depended upon an increase in AMP, ADP, or ATP since interconversion of these compounds was known to occur.

These experiments indicated that 3.09 μmoles of hypoxanthine were recovered from its control system and converted to 3.34 μmoles of IMP in the test system. The disappearance of adenine was balanced by the recovery of adenine-containing compounds. The compound represented by Ad-X was not identified. It was eluted between

TABLE II

FATE OF HYPOXANTHINE AND ADENINE IN BACTERIAL EXTRACT
Experimental procedures described in text.

Compound isolated	*Control μmoles*	*Test μmoles*	*Compound isolated*	*Control μmoles*	*Test μmoles*
Hypoxanthine	3.09	0	Adenine	6.35	0.36
AMP	0.19	1.52	AMP	3.16	6.15
IMP	0	3.34	Adx	1.55	3.33
ADP	0	1.57	ADP	1.70	2.43
ATP	4.3	1.34	ATP	1.86	1.80
Total	7.58	7.77		14.62	14.07

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AMP and ADP, and its absorption of u.v. light was maximum at 260 mμ. Only 1 μmole of pentose was present for each μmole of adenine if the compound had the same molar absorption coefficient as AMP.

From these results one may conclude that hypoxanthine and adenine were converted to their respective ribotides in the bacterial extract by combining with PRPP. Phosphoribosyl pyrophosphate was not added to the systems as a substrate but was assumed to be formed according to reaction (1) of this section.

DISCUSSION

Experiments which were designed to demonstrate the route of AICAR synthesis indicated that known purine precursors were not utilized for the production of AICAR in the bacterial extract. While this work was in progress, MOYED AND MAGASANIK[3] clarified the problem by demonstrating that the AICAR was a by-product of histidine synthesis. The present report indicated that the same route was followed in the *E. coli* extract. Therefore, the liberation of AICA-derivatives by bacterial whole cells served as an index to purine synthesis[5], but in the bacterial extracts the synthesis of AICAR served as an index to histidine synthesis.

The amount of AICAR synthesized in the minimal medium was considerably lower than that obtained in a complete medium. This would tend to indicate that AICAR was produced *de novo*, as in the synthesis of purines, as well as from AMP in the synthesis of histidine. All attempts to demonstrate the *de novo* route were unsuccessful. The other known alternative would depend upon a sequence of reactions involving the deamination of AMP to IMP followed by formate exchange reactions with glycine or glycinamide ribotide[18] to yield AICAR. Attempts to measure AMP-deaminase activity in the bacterial extracts by the spectrophotometric method[19] were unsuccessful.

The demonstration of mononucleotide synthesis in the bacterial extract involved the reaction of a purine base with a ribosephosphate derivative, presumably PRPP. The demonstration of this reaction supported the hypothesis advanced by GOTS[8] that the feed-back inhibition of purines might be the result of a shunting mechanism involving the PRPP. Phosphoribosylpyrophosphate (PRPP) was a common, obligate, intermediate in the *de novo* synthesis of purines[2] and of histidine[4]. When purines were added to bacterial whole cells[8], purine synthesis was inhibited. When purines were added to the bacterial extracts, histidine synthesis was inhibited as indicated by the inhibition of AICAR production. The feed-back inhibition mechanism is thought to be based on the shunting of PRPP from these routes into direct incorporation in purines to form nucleotides. Fig. 4 illustrates this probability. In the absence of added purines the *de novo* route for purine synthesis would be operative in the whole cells, and histidine synthesis would be operative in the extracts.

Only 10 μg/ml of adenine was required to exert almost complete inhibition of purine synthesis in the bacterial whole cells[8]. This would tend to indicate that the feedback mechanism would involve more than just the depletion of PRPP from the whole cells. Actually, one would expect smaller concentrations to be inhibitory in the whole cells than in the extracts since the reserve of ATP in the whole cells, which was required for the synthesis of PRPP, would be lower than that which could be added to a bacterial extract. Only 65 mμmoles of AICA-derivatives were synthesized by the whole cells and 74 mμmoles of adenine was found to be inhibitory for the reaction,

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indicating that the amount of PRPP that could be utilized for AICAR synthesis was incorporated into mononucleotides.

Other conservatory mechanisms were known to be operative in bacterial cells, as was demonstrated with valine[20] and pyrimidine[21] auxotrophs. One type of mechanism involved the inhibition of synthesis of the end product. Another known mechanism involved the inhibition of enzyme synthesis by the end product[4,22]. Purines were able to regulate purine and histidine synthesis by the former method.

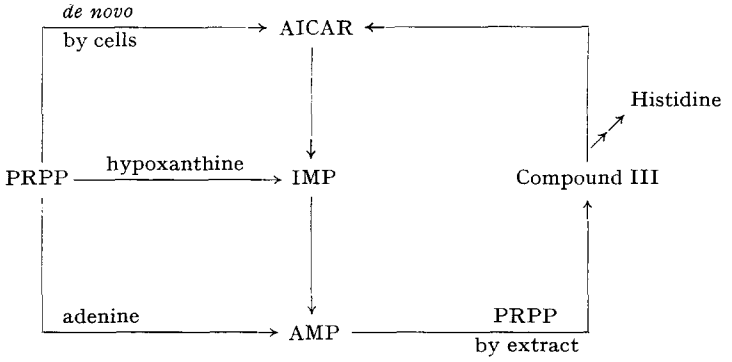

Fig. 4. The regulation of purine and histidine synthesis by purines.

ACKNOWLEDGEMENTS

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