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FORMATION OF 5-AMINOIMIDAZOLE RIBOSIDE BY ESCHERICHIA COLI: EVIDENCE FOR ITS STRUCTURE AND METABOLIC RELATIONSHIP TO THE PURINES

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SUMMARY

The arylamine accumulated by the mutant of *Escherichia coli* K-12, strain W-11, has been identified as 5-aminoimidazole riboside. The compound, isolated by an improved method, yielded upon chemical degradation glycine, formate and pentose in the expected proportions. The product contained no phosphorus. The arylamine was formed in microorganisms from [^{14}C]labeled formate or glycine and could be converted into inosinic acid by an enzyme system of pigeon liver. Chemical conversion of radioactive arylamine to the ureido derivative, hydrolysis to the free base and addition of carrier 5-ureidomidazole, yielded a picrate of 5-ureidoimidazole which could be crystallized to a constant specific activity.

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INTRODUCTION

The accumulation of a pentose-containing arylamine, thought to be 5-aminoimidazole riboside, by a purine-requiring mutant of *Escherichia coli* K-12, strain W-11, was reported by LOVE AND GOTS[1]. More recently, LEVENBERG AND BUCHANAN[2,3] described the biosynthesis of 5-aminoimidazole ribotide by a soluble enzyme system of pigeon liver. The ribotide derivative was isolated as a barium salt and was shown to be an intermediate in the synthesis of inosinic acid *de novo*.

The lability of the riboside derivative, as liberated by the bacterial mutant, hindered its isolation and identification. This report presents an improved method of synthesis and isolation, as well as additional studies on the properties and identity of the arylamine which substantiate the belief that the amine produced is 5-aminoimidazole riboside.

MATERIAL AND METHODS

The mutant of *Escherichia coli* and the basic synthetic medium used were the same as previously described[1].

The methods applied for the chemical analysis of the riboside derivative were identical to those described for other purine intermediates[3,4]. The PAULY diazo reaction was carried out according to the modification of KOESSLER AND HANKE[5]. Arylamine compounds were measured by the method of BRATTON AND MARSHALL[6], the final volume being 5.2 ml. Dowex 50 in the ammonium or potassium form (200–400 mesh) was used in the ion exchange procedures.

RESULTS

Arylamine synthesis

The cell yield could be increased by culturing the organisms in the presence of a higher purine concn. without the synthesis of the amine being affected. The relationship between the organism's ability to synthesize the amine and the concn. of purine on which it has been cultured was studied. The growth response of the organisms in the presence of various concns. of hypoxanthine is illustrated in Fig. 1. The cells which had been cultured on hypoxanthine concn. of 14 μg/ml exhibited maximum synthesis of the amine (Table I). For subsequent studies, strain W-11 that had grown without aeration was used as seed (1:30) to inoculate the synthetic medium containing 14 μg/ml of hypoxanthine and 0.2 % glucose. The cells were grown under intense forced aeration by bubbling air through the medium and were harvested after 11 h. The cells produced from one liter usually sufficed to give a concn. of 0.5 mg/ml when resuspended in 225 ml of medium. The final suspension for amine synthesis was supplemented with 0.2 % glucose and 1 μmole/ml of glycine, sodium formate, and glutamine; no purine was added to this suspension. Maximum synthesis of the amine occurred within 3 h, again with the aid of intense aeration. Longer periods of incubation usually resulted in the development of a yellow pigment and in a decrease of arylamine. The cells were removed by centrifugation. By the amount of pentose present and the color reaction of the arylamine, 40 to 60 μg/ml of the amine were present in the supernatant solution as a result of the biosynthetic process.

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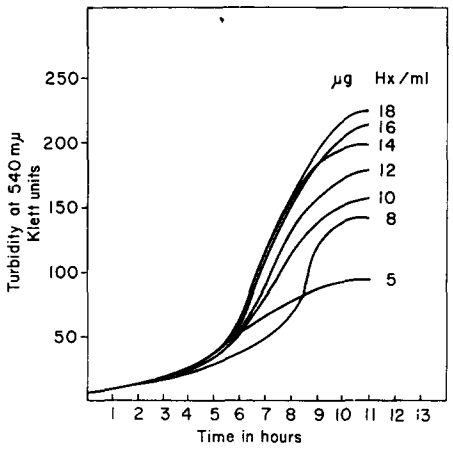

Fig. 1. Growth response of *Escherichia coli*, strain W-11, to various concns. of hypoxanthine. To each system consisting of 30 ml of synthetic medium containing 0.2% glucose and the various concns. of hypoxanthine, a suspension of *E. coli*, strain W-11, was added. The systems were incubated in 125-ml Erlenmeyer flasks, plugged with cotton, at 37° on a mechanical shaker.

TABLE I

ARYLAMINE SYNTHESIS BY *Escherichia coli*, STRAIN W-11

KLETT units/ml at 500 mμ.

Escherichia coli, strain W-11, which had been cultured in synthetic medium containing various concn. of hypoxanthine (see Fig. 1), were harvested after 11 h. The cells from the different systems were collected separately by centrifugation, washed with distilled water, and resuspended (0.5 mg/ml) in synthetic medium containing 0.2% glucose and 50 μg/ml of glycine, but no hypoxanthine. The suspensions (9.4 ml) were incubated in 50-ml Erlenmeyer flasks on a mechanical shaker at 37°. Aliquots of 1 ml were removed at the times indicated for the determination of arylamine by the BRATTON AND MARSHALL reaction.

Grown on hypoxanthine μg/ml	*Time in hours*				
	2.5	*3*	*3.5*	*4*	*4.5*
6	540	715	760	840	820
10	600	840	920	960	950
12	730	910	925	960	950
14	740	910	975	1090	1140
16	715	755	775	840	765
18	650	750	768	778	748

Arylamine isolation by ion exchange

After incubation, 20 ml of the final supernatant solution were adsorbed without adjustment of the pH on columns of Dowex 50 in the ammonium form (0.8·16 cm). After adsorption, the flow rate was adjusted to approx. 0.7 ml/min by means of air pressure.

The two methods of elution used gave identical results with regard to the diazotizable arylamines separated and with the chemical analysis of the amine in question. In one method ammonium hydroxide was used as the eluant and in the other sodium acetate buffers were applied.

Method A: Elution was begun with 50 ml of 0.0025 *M* sodium acetate, pH 5.5,

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followed by 0.01 *N* NH_4OH. A compound which failed to adsorb on the column, or which was eluted in the early fractions, reacted with the BRATTON AND MARSHALL reagents to yield a chromophore with an absorption maximum at 520 mμ. The first 15 ml of the alkaline fractions contained a second diazotizable arylamine whose chromophore had an absorption maximum at 500 mμ identical to that of the original supernatant fluid and to that of 5-aminoimidazole. When more than one column was used during the isolation procedure, the first 15 ml of the alkaline fractions were collected from each column and pooled. The pooled fractions were lyophilized to dryness. The lyophilized compound decomposed slowly at —20°.

Method B: Two diazotizable amines were also eluted by sodium acetate buffers (Fig. 2). The order of elution corresponded to that obtained with NH_4OH. No apparent advantage was gained by increasing the volumes of the buffers before the elution with unbuffered sodium acetate. The recovery of total diazotizable amine ranged from 83 to 94 %. The second amine, which was thought to be 5-aminoimidazole riboside, accounted for approx. 83 % of the total amines eluted.

Chemical analysis

The second diazotizable amine from each type of column was analyzed for pentose, glycine, formate, acid hydrolyzable ammonia, and phosphorus (Table II). If the base moiety were 5-aminoimidazole[7], the corresponding ratios 1:1:1:2:0 would be expected. Although most of the contaminating inorganic ammonia could be removed during lyophilization, the amount of residual ammonia was too great to permit accurate acid-labile ammonia determinations. The other ratios found agreed with the conclusion that the base moiety was 5-aminoimidazole and that the arylamine was the riboside derivative.

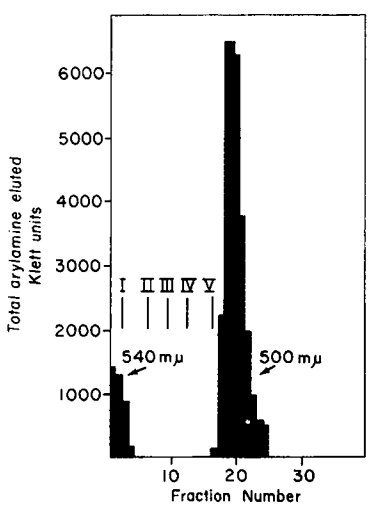

Fig. 2. Ion exchange of arylamine as produced by *Escherichia coli*, strain W-11. Procedures before elution are described in the text. The eluting fluids were: I = 0.0025 *M* Na-acetate buffer, pH 5.6 (40 ml); II = 0.005 *M* Na-acetate buffer, pH 5.6 (40 ml); III = 0.025 *M* Na-acetate buffer, pH 5.6 (40 ml); IV = 0.05 *M* Na-acetate buffer, pH 5.6 (50 ml); V = 0.20 *M* Na-acetate, not buffered (150 ml). The chromophores produced by the reaction of the arylamines with the BRATTON AND MARSHALL reagents were measured in the Klett-Summerson colorimeter at the indicated wave lengths.

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TABLE II

CHEMICAL ANALYSIS OF THE ARYLAMINE PRODUCED BY *Escherichia coli*, STRAIN W-11

Pooled fractions of the arylamine isolated by the described ion-exchange methods were analyzed by the method of HARTMAN, *et al.*[4]. The expected values are based on those obtained by HUNTER AND NELSON[7] during the analysis of 5-aminoimidazole.

Method of isolation	*Molar ratios*				
	Glycine	*Pentose*	*Formate*	*Phosphorus*	*NH_3*
NH_4OH method	0.94	1.0	1.12	none	too high
Na-acetate method	0.99	1.0	1.19	none	too high
Average values	0.96	1.0	1.15	none	
Expected values	1.0	1.0	1.0	none	2.0

Since the elution with NH_4OH was accomplished more rapidly and with greater ease than that with sodium acetate, and since the chemical constituents of the amines were similar, the second amine eluted with NH_4OH was used in subsequent studies.

Metabolic precursors of the arylamine

When [^{14}C]sodium formate and [1-^{14}C]glycine were added to the final incubation mixture, they were readily incorporated into the arylamine; [^{14}C]bicarbonate was not incorporated under the same conditions. The amount of incorporation of radioactive formate and glycine was compared with the concn. of pentose. It was found that the quantities of radioactive substrates incorporated into the arylamine (Table III) were less than 1:1 (substrate/amine), probably because of the dilution of the exogenous radioactive substrates by metabolites produced endogenously.

TABLE III

THE INCORPORATION OF [^{14}C]SODIUM FORMATE AND [1-^{14}C]GLYCINE INTO ARYLAMINE

The arylamine was synthesized according to the final standardized procedure with the appropriate substitution of radioactive substrate.

Radioactive substrate	*counts/min/μmole*	*Arylamine μmole/ml*	*Arylamine counts/min/μmole*	*Incorporation %*	*Average % Incorporation*
[^{14}C]formate	(29,800)	0.254	17,100	57	62.5 (4)*
[1-^{14}C]glycine	(20,400)	0.224	13,500	66	65.5 (2)

* Number of expts.

Relationship of the arylamine to purine biosynthesis

Formate is known to be the carbon source of positions 2 and 8 of the purine ring[8]. If the arylamine were metabolically related to an intermediate of purine biosynthesis, the [^{14}C]aminoimidazole riboside synthesized by *E. coli* from [^{14}C]formate might be expected to be labeled in the 2 position and the radioactivity of this compound, on conversion to a purine, to be found solely in the 8 position of the ring of the latter structure (Fig. 3).

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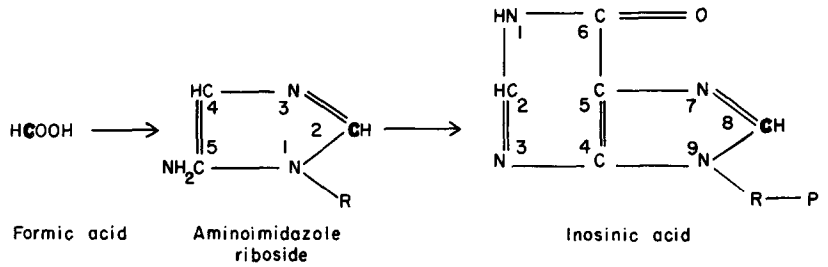

Fig. 3. Conversion of [2-^{14}C]5-aminoimidazole riboside to inosinic acid. Experimental conditions described in text.

[^{14}C]arylamine was synthesized in the presence of [^{14}C]sodium formate and isolated by the NH_4OH method. When this arylamine (55,000 counts/min/μmole) was incubated with pigeon liver extract in the presence of unlabeled formate, significant amounts of the radioactive substrate (25 %) were converted into radioactive inosinic acid[2]. Thus the arylamine is definitely involved in the biosynthesis of inosinic acid in the pigeon liver system. The inosinic aced obtained was converted to uric acid by the method of SCHULMAN AND BUCHANAN[9], 50 mg of uric acid were added as carrier, and the uric acid was degraded chemically[8] to yield fractions representing carbon atoms 2 and 8 separately. Carbon atom 8 was found to contain 96 % of the radioactivity of the entire molecule, which indicated that it alone had been derived metabolically from the radioactive carbon of the arylamine. This expt. supported the conclusion that the compound could be used for purine synthesis and that it was not degraded before its conversion. The riboside derivative presumably was converted to its ribotide before inosinic acid synthesis[10].

Conversion of arylamine to its ureido derivative

The observation that radioactive substrates may be incorporated into the arylamine provided another means of identifying the compound. Arylamine synthesized by *E. coli*, strain W-11, in the presence of radioactive formate was isolated and lyophilized to dryness. From the amount of pentose present, the sample contained approx. 40 μmoles of the amine. This was dissolved in absolute methanol, the insoluble debris sedimented by centrifugation, and the ureido derivative of the arylamine was prepared in the same manner as for 5-aminoimidazole ribotide[3]. The decrease in the intensity of the PAULY reaction showed that approx. 40 % of the arylamine could have reacted to form the ureido derivative. The ureidoimidazole picrate, which was made as previously described[3], was plated for determination of radioactivity and was recrystallized before each succeeding determination. The samples had specific activities of 2,890, 2,590 and 2,670 counts/min/mmole on successive recrystallizations. The constancy of these activities provided additional evidence that the arylamine was a derivative of 5-amino-imidazole.

DISCUSSION

The ion exchange procedures used facilitated the isolation of the relatively pure amine in high yield. If the adsorption of the amine on the column was delayed, the amount of 5-aminoimidazole riboside recovered decreased while the amount of

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amine in the first fraction increased. This tended to indicate that the amine whose chromophore absorbed at 520 mμ was a derivative of 5-aminoimidazole riboside. No attempt was made to obtain supporting data.

Contaminating ammonia prohibited accurate nitrogen determinations but the other evidence for the structure, *i.e.*, (a) the production of a chromophore with maximum absorption at 500 mμ, (b) the chemical analysis of the basic components, (c) the incorporation of [^{14}C]glycine and formate, (d) the biochemical conversion of the amine to inosinic acid, and (e) the chemical formation of the ureido derivative, makes it highly improbable that the structure is other than that of 5-aminoimidazole riboside.

In contrast to the initial report of the recognition of the amine[1], the present improved procedures yielded a product which no longer exhibited absorption at 300 mμ. Most samples absorbed non-specifically in the lower wave lengths of the u.v. region and some of the isolated samples displayed a slight absorption maximum at 230 mμ. A yellow pigment slowly developed in the solutions concomitantly with a decrease in the diazotization reaction; its formation accounts for the discrepancy of the spectrophotometric results of the present and earlier expts.

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