Biosynthesis of Riboflavine by a Purine-Requiring Mutant Strain of *Escherichia coli*

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1. Riboflavine biosynthesis occurs in non-proliferating cultures of a purine-requiring strain of *Escherichia coli* (ATCC no. 13863). 2. No significant incorporation of radioactivity from [1-¹⁴C]glycine into either C-4a and C-9a of riboflavine or into nucleic acid purines is detected under the above conditions; appreciable incorporation of label into 5-aminoimidazole-4-carboxamide occurs. However, the label of [6-¹⁴C]guanine is incorporated significantly into C-4 of riboflavine and into nucleic acid adenine and guanine; the specific radioactivity of the riboflavine is approximately twice that of either adenine or guanine of nucleic acid. 3. These results show that a purine derivative is an obligatory intermediate in riboflavine biogenesis.

Evidence has been accumulated to support a hypothesis for the direct involvement of purines in riboflavine biosynthesis (for review see Plaut, 1961a,b). The direct convertibility of the major portion of the purine skeleton into rings B and C of riboflavine has been demonstrated (McNutt, 1956, 1960; Audley, Goodwin & McEvoy, 1959; Audley & Goodwin, 1962). However, the question raised by Plaut (1961a), namely whether or not a purine derivative is an obligatory intermediate in the biosynthesis of the vitamin, has not been answered. The available evidence may be interpreted to support either of two pathways:

Wilson & Pardee (1962) demonstrated uninterrupted biosynthesis of riboflavine in *Escherichia coli* after inhibition of growth by various means, e.g. ultraviolet irradiation of the organisms, addition of inhibitors (5-methyltryptophan, chloromycetin etc.) or by removal of the exogenous purine supply from purine-less mutants. With the purine-requiring mutants, the question arises whether there is an obligatory involvement of

purine derivatives in riboflavine biosynthesis. Thus either the endogenous purine derivatives of the cells might be utilized for riboflavine biosynthesis, or alternatively pathway (2) may function.

The present work was undertaken to determine whether or not the purine moiety is an obligatory intermediate in riboflavine biosynthesis.

EXPERIMENTAL

Materials. The following materials were purchased from the suppliers indicated: acid alumina AG4 (chromatographic aluminum oxide; 200–400 mesh), cation-exchange resin (Dowex) AG50W (X8; H⁺ form; 200–400 mesh), DEAE-cellulose (Cellex-D; 0.78m-equiv./g.) and [6-¹⁴C]guanine (15mc/m-mole) from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; Lloyd's reagent from Hartman–Leddson Co., Philadelphia, Pa., U.S.A.; casein hydrolysate (acid hydrolysate; vitamin-free and salt-free) from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; riboflavine (U.S.P. grade) from Merck and Co. Inc., Rahway, N.J., U.S.A.; [1-¹⁴C]glycine (1mc/m-mole) from Volk Radiochemical Co., Chicago, Ill., U.S.A.; 5-aminoimidazole-4-carboxamide (A grade) and 5-amino-1-(p-ribosyl)imidazole-4-carboxamide (A grade) from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

Lumiflavine was synthesized by the procedure of Kuhn & Reinemund (1934).

Organism. *Escherichia coli* B-96/1t (ATCC no. 13863) was used. This mutant was derived from the purine-less mutant *E. coli* B-96; in addition to purine it requires tryptophan for growth (Gots, 1957). Thus the incorporation of labelled purine into riboflavine could be investigated under conditions in which no growth occurs by withdrawing

tryptophan from the medium. The organism accumulates 5-amino-1-(α -ribosyl)imidazole-4-carboxamide (Gots, 1957).

Media and conditions of culture. The minimal medium for growth consisted of the minimal salts solution of Davis & Mingioli (1950) supplemented with glycerol (2mg./ml.), casein hydrolysate (2mg./ml.), adenine (20 μ g./ml.) and tryptophan (20 μ g./ml.). All flasks were incubated at 37° with shaking on a Gyrotory water bath shaker (New Brunswick Co. Inc., New Brunswick, N.J., U.S.A.). Growth was determined by the absorption of light at 660m μ (Klett-Summerson colorimeter, filter no. 66) and by protein assay (Lowry, Rosebrough, Farr & Randall, 1951). Under these conditions a colorimeter reading of 70 was equivalent to 28 μ g. of protein/ml.

The micro-organism was maintained on agar slants of a complete medium (Lederberg, 1950) containing tryptophan (20 μ g./ml.). The stock culture was stored at 5° and sub-cultured at monthly intervals.

Each experiment was performed in the following manner. A mother culture was grown in 10ml. of the minimal medium. The cells were harvested, washed with sterile 0.9% NaCl and resuspended in 0.9% NaCl to obtain a suspension transmitting 90% of the incident light, in a Klett-Summerson colorimeter, filter no. 66. Then 3 drops of the suspension were added to each of three 1l. Erlenmeyer flasks each containing 450ml. of minimal medium. The cells were harvested towards the end of the exponential growth phase, combined, washed with 0.9% NaCl and resuspended in fresh warm minimal medium lacking either one or both of the essential compounds required for growth. These manipulations were conducted aseptically.

In the experiments described in Fig. 1, 15ml. samples for analysis were taken aseptically at various time-intervals. A 12ml. portion of each sample was centrifuged and both cell residue and supernatant liquid were retained. The supernatant liquid was made 0.1N with respect to HCl and allowed to stand for 2 days at 37° to hydrolyse any FAD present to FMN. The residue of the cell suspension was extracted with 1ml. of cold 5% (w/v) trichloroacetic acid. The flavines present in the trichloroacetic acid extract of the cells and in the acidified supernatant layer were determined.

In the experiments with 14 C-labelled compounds (Table 1), the cells were resuspended in fresh minimal medium lacking both tryptophan and purine, and sterile [6- 14 C]-guanine or sterile [1- 14 C]glycine was added. Samples for analysis were taken at zero time and after 8hr. of incubation. The reactions were stopped by the addition of trichloroacetic acid to give a final concentration of 5%. The cell residue was collected by centrifugation, washed with 5ml. of cold 5% (v/v) acetic acid and assayed for the specific radioactivity of the nucleic acid purines. The supernatant trichloroacetic acid solution was incubated overnight at 37° and riboflavine was assayed, isolated and degraded.

Isolation of flavines. The flavines, FMN and riboflavine, were concentrated from the 5% trichloroacetic acid extracts by column chromatography on Lloyd's reagent. Columns (1cm. $\times$ 8cm.) of pretreated Lloyd's reagent were prepared as described by Plaut (1963). The columns were washed with 5% acetic acid solution (100ml.). After application of the samples, the columns were washed with 5% acetic acid (100ml.), deionized water (100ml.) and a solution of 0.5% pyridine in 2% (v/v) acetic acid (100ml.). The FMN and riboflavine were eluted with an aqueous solution

containing pyridine (25%, v/v) and acetic acid (2%, v/v). The solution of flavines was concentrated to dryness in a vacuum and the residue was dissolved in 10ml. of 0.01N-HCl.

The solution from above was neutralized with N-NaOH and applied to a column (0.7cm. $\times$ 10cm.) of washed DEAE-cellulose (acetate form). FMN was retained as a narrow band at the top of the column, whereas riboflavine emerged in the effluent and was removed completely from the DEAE-cellulose by washing with 10ml. of water.

The effluent and wash fractions were combined, and the riboflavine contained was diluted approx. fourfold with non-radioactive carrier and was purified further by column chromatography on acid alumina (Plaut, 1963), a benzyl alcohol extraction step (Wacker, Harvey, Winestock & Plaut, 1964) and, after dilution with additional non-radioactive carrier, by recrystallization to constant specific radioactivity from HCl.

The chemical degradation of riboflavine was as described by Plaut (1954a).

Assay of riboflavine and degradation products. The riboflavine and degradation products obtained in the experiments with 14 C-labelled compounds were assayed spectrophotometrically or gravimetrically. The absorption coefficient for lumiflavine reported by McNutt (1956) was used for its determination.

Where smaller concentrations of riboflavine were assayed, a fluorimetric technique was used. Thus 0.7ml. portions of the trichloroacetic acid extracts of the pellets of cells (see sampling procedure under Media and conditions of culture) were adjusted to pH6.9 with 0.35ml. of 2.0M-KH₂PO₄ buffer, pH6.9; 3ml. portions of the acidified culture medium fraction were adjusted to pH6.9 with 1.5ml. of the 2.0M-phosphate buffer. The fluorescence of these neutralized samples was compared with a reagent blank and a standard riboflavine solution by using a Farrand model A fluorimeter, as described by Plaut (1963).

Isolation and purification of nucleic acid purines. The cell debris precipitated by cold trichloroacetic acid was extracted with 10–15ml. of 5% trichloroacetic acid for 15min. at 90–95° (Schneider, 1945). The residue was removed by centrifugation and washed with 4–5ml. of hot 5% trichloroacetic acid. The trichloroacetic acid contained in the combined supernatant solution was removed by ether extraction, and the volume was reduced under a vacuum to 3ml. The solution was made 1N with respect to HCl and complete hydrolysis was ensured by heating on a boiling-water bath for 90min. (Lepage & Heidelberger, 1951). The hydrolysate was adjusted to 2N with respect to HCl and the purines were chromatographed on a column (0.7cm. $\times$ 8cm.) of Dowex AG50W (X8; H⁺ form) with 2N-HCl according to the method of Cohn (1955). The eluted purines, adenine and guanine, were purified further by isolation as their silver salts (Loring, Fairley, Bortner & Seagran, 1952).

Adenine and guanine were assayed spectrophotometrically by using the molecular extinction coefficients tabulated by Burton (1962).

Isolation of 5-aminoimidazole-4-carboxamide. A 200ml. portion of the initial effluent from the column of Lloyd's reagent (see under Isolation of flavines) was freeze-dried. The residue was dissolved in 100ml. of water and the resulting solution was adjusted to pH1.8–2.0 with N-HCl. This solution was applied to a column (1cm. $\times$ 10cm.) of Dowex AG50W (X8; H⁺ form) that had been equilibrated with

0.01N-HCl. After a washing with 250 ml. of water, 5-aminoimidazole-4-carboxamide was eluted from the column with 100 ml. of 0.1N-NH₃. The solution containing 5-aminoimidazole-4-carboxamide was freeze-dried, and the material was dissolved in water and assayed by a modification of the Bratton-Marshall test (Lukens & Flaks, 1963). The 5-aminoimidazole-4-carboxamide was purified further by descending paper chromatography in the 'diethylene glycol system' [butan-1-ol-diethylene glycol-water (4:1:1, by vol.) saturated with boric acid in the trough; 1N-NH₃ in the tank; Whatman 3MM paper]. 5-Aminoimidazole-4-carboxamide was eluted from the paper with water by Dent's (1947) procedure, assayed, then rechromatographed in the 'propanol system' [propan-1-ol-water (4:1, v/v); descending technique; Whatman 3MM paper]. The 5-aminoimidazole-4-carboxamide was eluted again and re-assayed.

5-Aminoimidazole-4-carboxamide (or its ribosyl derivative) was located on the paper by quenching of fluorescence of ultraviolet light or by spraying with Bratton-Marshall reagent (Lukens & Flaks, 1963). Its identity was confirmed by comparison of the position of the spots on the chromatogram with that of the authentic substance.

Determination of radioactivity. The counting techniques employed were as described by Plaut (1963).

RESULTS

Production of riboflavine by *E. coli* B-96/1t. The results described in Fig. 1 show that riboflavine production per cell unit proceeds under conditions where no growth could be detected (i.e., without added purine and/or tryptophan) at apparently the same rate as when growth occurs (purine and tryptophan present). The absence or presence of added purine did not appear to influence flavine production.

Experiments with [1-¹⁴C]glycine and [6-¹⁴C]guanine. The incorporation of radioactivity from [1-¹⁴C]glycine or [6-¹⁴C]guanine into riboflavine was investigated under conditions where essentially no proliferation of the micro-organism occurred. It was shown that some radioactivity from [1-¹⁴C]glycine was incorporated into riboflavine (Table 1). However, since most (approx. 70%) of this label was recovered in the chemical degradation product, *N*-methyl-4,5-dimethyl-*o*-phenylenediamine, which represents the *o*-xylene fragment and C-1' of the vitamin, only a minor portion (if any) of this label could have been incorporated into C-4a and C-9a of the riboflavine molecule; radioactivity from this amino acid was found to be incorporated exclusively into these positions in previous studies (Plaut, 1954a; see also Goodwin & Jones, 1956). Presumably, in the present experiment, a radioactive metabolic degradation product of glycine was incorporated into the flavine molecule in a portion other than the pyrimidine moiety (e.g., see Plaut, 1954b; Plaut & Broberg, 1956). The micro-organism can utilize this amino acid, since 5-aminoimidazole-

Fig. 1. Synthesis of flavines by *E. coli* B-96/1t in the presence and absence of a purine and tryptophan. The conditions of incubation of the cultures and the procedures for the assay of total flavines and for the assay of protein are described in the text. Cells grown in minimal medium were harvested, washed with sterile 0.9% NaCl and resuspended in fresh medium (150 ml. in each 500 ml. flask) to give suspensions, each of which gave a colorimeter reading of 70 (Klett-Summerson colorimeter, filter no. 66). ●—●, Complete minimal medium (see the text) (growth occurred); no growth occurred in minimal media from which the following components had been deleted: Δ....Δ, purine; □----□, tryptophan; ○---○, purine and tryptophan.

4-carboxamide recovered from the cultures had a specific radioactivity more than half of that of the added glycine, indicating that the purine biosynthetic pathway up to the biochemical block was functioning.

In contrast with the experiments with [1-¹⁴C]glycine, appreciable incorporation of radioactivity from [6-¹⁴C]guanine into riboflavine occurred, and over 90% of the label derived from the purine was recovered in the expected position, i.e. C-4 of riboflavine (Table 1). In the same experiment, significant incorporation of the label from guanine could be detected equally in adenine and guanine isolated from the nucleic acid fraction. This indicated that, though there was no detectable

Table 1. *Incorporation of [1-¹⁴C]glycine and [8-¹⁴C]guanine into riboflavin, nucleic acid purines and 5-aminoimidazole-4-carboxamide by E. coli B-96/1t*

Labelled glycine or guanine was added to suspensions of cells (colorimeter reading 70, as determined in a Klett-Summerson colorimeter, filter no. 66) in 450 ml. of medium, and incubated in 11. flasks for 8 hr. under the conditions described in the text. The isolation and determination of riboflavin, nucleic acid purines, 5-aminoimidazole-4-carboxamide, and the degradation of riboflavin, are described in the Experimental section.

Compound or carbon atoms	[1- ¹⁴ C]Glycine added		[6- ¹⁴ C]Guanine added	
	Specific radioactivity (counts/min./μmole)	Total radioactivity (counts/min.)	Specific radioactivity (counts/min./μmole)	Total radioactivity (counts/min.)
Glycine (added)	1.6 × 10 ⁵ **	1.01 × 10 ⁸	—	—
Guanine (added)	—	—	4.55 × 10 ⁵	3.18 × 10 ⁶
Riboflavin (recovered)	6.7 × 10 ²	1.11 × 10 ²	1.35 × 10 ⁵	4.56 × 10 ³
Degradation of riboflavin:				
Lumiflavin	6.4 × 10 ²	32	1.23 × 10 ⁵	6.56 × 10 ²
C-5 + C-6 + C-7 + C-8 + C-8a + C-10a + C-1*	4.4 × 10 ²	8	—	—
C-4 + C-5 + C-6 + C-7 + C-8 + C-9a + C-4a + C-8a + C-10a + C-1†	—	—	9.93 × 10 ⁴	3.04 × 10 ²
C-5 + C-6 + C-7 + C-8 + C-9a + C-4a + C-8a + C-10a + C-1‡	—	—	4.26 × 10 ³	8
C-2§	—	—	2.23 × 10 ²	0.5
C-4	—	—	1.25 × 10 ⁵	2.97 × 10 ²
5-Aminoimidazole-4-carboxamide	8.7 × 10 ⁴	4.0 × 10 ⁵	—	—
Adenine	17	7	5.57 × 10 ⁴	8.74 × 10 ⁴
Guanine	5	3	6.06 × 10 ⁴	17.7 × 10 ⁴

* N-Methyl-4,5-dimethyl-o-phenylenediamine.

† 6,7-Dimethyl-2-oxo-quinoxaline-3-carboxylic acid.

‡ 6,7-Dimethyl-2-oxo-quinoxaline.

§ Dioxanthylurea.

|| Carbon dioxide.

** Calculated for dilution by non-radioactive glycine in the medium.

growth under the incubation conditions used, turnover of nucleic acids and interconversion of guanine and adenine occurred. The specific radioactivity of riboflavine was approximately twice as great as that of the purines isolated from the nucleic acids, suggesting that the purine derivative precursor is utilized for riboflavine biosynthesis before its incorporation into nucleic acids.

DISCUSSION

The present results demonstrate that the biosynthesis of riboflavine can occur in the absence of growth in cultures of a purine-requiring strain of *E. coli*, in confirmation of the observations of Wilson & Pardee (1962). However, the net synthesis of flavines observed does not involve the utilization of simple precursors for the synthesis of either a purine derivative or flavines, since radioactivity from [1-¹⁴C]glycine is not incorporated significantly into nucleic acid purines and the C-4a + C-9a portion of riboflavine, whereas appreciable amounts of label can be recovered in 5-aminoimidazole-4-carboxamide. The marked transfer of label from [6-¹⁴C]guanine into riboflavine demonstrates that the endogenous supply of purine derivatives (free purines, purine nucleosides, purine nucleotides etc.) is the source of precursors for the observed net formation of flavines.

The biochemical block in purine biosynthesis in *E. coli* B-96/1t is located between 5-amino-1-(5'-phospho-D-ribosyl)imidazole-4-carboxamide and inosinic acid (Gots, 1957). If an intermediate serves as a common precursor of purines and of riboflavine, as visualized in pathway (2), it must be 5-formamido-1-(5'-phospho-D-ribosyl)imidazole-4-carboxamide. However, this is unlikely since the free formamido derivative does not appear to accumulate as a product of the 5-amino-1-(5'-phospho-D-ribosyl)imidazole-4-carboxamide-transformylase reaction. Indeed, the formylating enzyme has not been separated from the subsequent cyclizing step, and the transformylase and inosinase activities may be properties of the same protein (Flaks, Erwin & Buchanan, 1957). This view has been supported by the observation that a loss of inosinase and transformylase activities was caused apparently by a single mutation in several organisms (Levin & Magasanik, 1961). Thus it may be concluded from the present studies that a purine compound is an obligatory intermediate in riboflavine biosynthesis in *E. coli*, and possibly in other organisms.

The present studies give little information about the level at which a purine derivative is utilized

for riboflavine biosynthesis. However, the results demonstrate that the diversion of such a substance to riboflavine synthesis must occur at the level of inosinic acid or later in the scheme of purine biosynthesis.

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