ISOLATION OF 4-RIBITYLAMINO-5-AMINO-2,6-DIHYDROXYPYRIMIDINE FROM A HIGH FLAVINOGENIC MOLD
EREMOTHECIUM ASHYII1

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Summary The addition of glyoxal, a trapping agent, caused simultaneoulsy the accumulation of a green fluorescent compound and the inhibition of riboflavin formation in non-growing cells of Eremothecium ashbyii. The fluorescent compound purified was identified as 8-ribityllumazine from the results of spectrophotometric and fluorometric analyses. Accordingly, the fragment, except for the glyoxal portion on the 8-ribityllumazine molecule, 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine, is believed to be an intermediate in the riboflavin biosynthetic pathway.

Purines are known to be incorporated into the skeleton of riboflavin accompanied by the elimination of C(8) on the purine molecule (1–4). However, the pathway from the purine bases to 6,7-dimethyl-8-ribityllumazine on the riboflavin biosynthetic pathway is not known. Recently, Lingens and Bacher (5,6) isolated some green fluorescent compounds from riboflavin-deficient mutants using trapping agents (glyoxal, diacetyl), which easily bind with the expected intermediate in flavinogenesis to give the corresponding pteridine. They suggested that one of the intermediates is 4-ribitylamino-2,5-diamino-6-hydroxypyrimidine and that the other is 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine based on the structure of the pyrimidine portions of the isolated compounds. However, equivocal points remain, especially in the structure of the latter compound.

In the present paper, the possibilities of these pyrimidines being intermediates in flavinogenesis were examined using a trapping agent, glyoxal, in non-growing

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cells of a high flavinogenic mold *Eremothecium ashbyii*.

**MATERIALS AND METHODS**

Lumazines: 6,7-Dimethyl- and 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-lumazine were synthesized by a non-enzymatic reaction of the glyoxal or diacetyl monomer or dimer and 4,5-diamino-2,6-dihydroxypyrimidine which was synthesized by the method of Kuhn and Cook (7) in a weak acid solution with hydrochloric acid at 35°C for 27 hr. Pterins: 6,7-Dimethyl- and 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)pterin were synthesized by the reaction of the glyoxal or diacetyl monomer or dimer and 2,4,5-triamino-6-hydroxypyrimidine which was synthesized by the method of Rembold and Metzger (8) under the conditions described above. The lumazines and pterins synthesized were purified by column chromatography on DEAE-cellulose.

*Eremothecium ashbyii* cells (at the exponential phase) which had been cultivated for 1 day were harvested by filtration from the natural nutrients medium (9) and adequately washed with distilled water. The mycelia were suspended in 0.1 M phosphate buffer (pH 6.8) containing 0.5% glucose at a ratio of 20 ml buffer per 1 g mycelia (wet weights). The mycelial suspension was starved by incubating it at 27°C for 8 hr in the dark on a reciprocal shaker (62 oscillations/min). After the starvation, the mycelia were collected, washed and resuspended in the same buffer containing the test compound (glyoxal). The compound was infiltrated into mycelia under reduced pressure in a desiccator: The desiccator was connected to a vacuum pump for a short time and then open to the air. This process was repeated three times with a increasing period of evacuation; 2, 3, and 5 min for the 1st, 2nd, and 3rd cycle, respectively. The suspension was incubated for 18 hr on a reciprocal shaker (85 oscillations/min) under the conditions used for starvation (10).

After the incubation of the non-growing cells, the green fluorescent compound accumulated in the cells was extracted by heating a mixture of cells-distilled water (1:2.5, w/v) on a water bath at 80°C for 15 min. The extract (50 ml) obtained by centrifugation were made up to a 5% acetic acid solution, applied to the column (2×10 cm) on Lloyd’s reagent-cellulose (11), then eluted with 5% acetic acid at the rate of 20 ml/5 min. The green fluorescent fraction was collected after 10–20 min and treated with charcoal (9). The samples obtained were dissolved in water and applied to a column (1×18 cm) of Dowex 50W×4 (H+, 200–400 mesh). Chromatography was developed by elution with water at a rate of 3 ml/5 min. The green fluorescent fraction obtained after 20–60 min was again run through a column (1×20 cm) packed with DEAE-cellulose. A fluorescent compound was eluted after 130–150 min from the column with 0.008 N acetic acid at a rate of 3 ml/10 min. This was dried in vacuo. The green fluorescent compound in the non-growing cell medium underwent the same purification steps.
as the above after charcoal treatment.

To identify pteridine portion of the isolated compound, the purified compound was dissolved in water which was followed by photolysis at a cool temperature for 6 hr at a distance of 40 cm from a lamp (110 V–250 W) emitting visible light. The photolytic compound (de-ribitylated compound) was purified by column chromatography on DEAE-cellulose.

Paper chromatography was done in the dark by the ascending method at room temperature using various solvents. Fluorescent spots on the papers were localized under an ultraviolet light in the dark.

RESULTS

1. Effects of glyoxal on riboflavin and the accumulation of a green fluorescent compound

Figure 1 shows that added glyoxal markedly inhibits riboflavin formation with increased concentrations except in the range of 0.1–0.3% concentration. Riboflavin formation was rigidly inhibited over the 0–0.1% concentration range of glyoxal, indicating minimum values at a 0.1% concentration. However, accumulation of a green fluorescent compound was observed inside and outside

![Figure 1](image-url)  

Fig. 1. The inhibition of riboflavin production and the accumulation of a green fluorescent compound in the presence of glyoxal. Flavin was calculated from the absorbances at 450 nm using 11.2 as millimolar extinction coefficient of riboflavin. The amounts of flavin in the medium and mycelia were added after the incubation of non-growing cells and are expressed as total flavins. The amounts of green fluorescent compound were calculated as those of the 6,7-dimethyl-8-ribityllumazine from the fluorometric analysis. See details in MATERIALS and METHODS for the experiments with non-growing cells.
cells under the inhibition of riboflavin due to the addition of glyoxal. The fluorescent compound was accumulated noticeably in the range of 0-0.1% glyoxal with the maximum values of accumulation at a 0.1% concentration. Thus, a clear-cut correlation was observed between the inhibition of riboflavin formation and the accumulation of the green fluorescent compound at a 0-0.1% concentration of glyoxal.

In this case, the true amounts of the green fluorescent compound can be almost two times as much as the amounts shown in this figure because the fluorescence intensity of the fluorescent compound, if the compound is 8-ribityllumazine, is about one-half of that of 6,7-dimethyl-8-ribityllumazine.

2. Ultraviolet absorption, excitation and emission spectra of the photolytic compound and lumazine

As seen in Fig. 2, the ultraviolet absorption spectra of the de-ribitylated compound, derived by photolysis from the isolated compound, were identical with those of lumazine of the lumazines and pterins in the acid and alkaline solutions. Maximum absorptions were observed at 228 and 330 nm in 0.1 N H₂SO₄ and at 237, 271 and 350 nm in 0.1 N NaOH.

![Fig. 2. Ultraviolet absorption spectra of the photolytic compound and lumazine.](image)

Excitation spectra were obtained by exciting the test compounds with the wavelengths given in Fig. 3 and determining the fluorescence emitted at 530 nm. Figure 3 shows the good coincidence between spectra of the de-ribitylated compound and lumazine. Maximum absorptions were observed at 250, 280 and 330 nm in 0.05 M phosphate buffer (pH 7.0). The absorption maximum at 330 nm clearly differed from those of other lumazines and pterins.

Emission spectra were obtained by exciting the compounds with a wavelength of 300 nm and determining the fluorescence emitted at the wavelengths
Fig. 3. Excitation spectra of the photolytic compound and lumazine. Patterns were determined in 0.05 M phosphate buffer (pH 7.0). For experimental conditions see details in the text.

Fig. 4. Emission spectra of the photolytic compound and lumazine. Patterns were determined in 0.05 M phosphate buffer (pH 7.0). For experimental conditions see details in the text.

given in Fig. 4. The maximum absorptions of the de-ribitylated compound and lumazine were detected at 450 nm in 0.05 M phosphate buffer (pH 7.0).

3. Paper partition chromatography of the photolytic compound and lumazine in various organic solvents

As seen in Table 1, the \( R_f \) values of the de-ribitylated compound and lumazine

| Solvents* | A | B | C | D | E | F | G | H |
|-----------|---|---|---|---|---|---|---|---|
| Photolytic compound | 0.61 | 0.62 | 0.07 | 0.57 | 0.17 | 0.64 | 0.50 | 0.43 |
| Lumazine | 0.61 | 0.62 | 0.07 | 0.57 | 0.16 | 0.64 | 0.51 | 0.43 |

* A: BuOH:AcOH:H₂O (3:2:2), B: BuOH:Py: H₂O (6:4:3), C: t-BuOH: NH₄OH: H₂O (60:5:3), D: t-BuOH: NH₄OH: H₂O (60:5:35), E: BuOH: EtOH: 2N NH₄OH (20:2:5), F: t-BuOH: Py: H₂O (60:15:35), G: PrOH: NH₄OH: H₂O (60:3:1), H: t-BuOH: H₂O (60:40).
were the same on paper chromatograms in various solvent systems. In this experiment, the fluorescence of these compounds on the chromatograms was noticeably enhanced by ammonia vapor under ultraviolet light, as reported by Goodwin and Treble (12). Lumazines generally showed their characteristics in the ammonia vapor.

DISCUSSION

Ultraviolet absorption spectra of the isolated compound coincided well with those of the 8-ribityllumazine isolated by Lingens et al. (5). However, 8-ribityllumazine is almost similar to 6,7-dimethyl-8-ribityllumazine in its absorption spectra. Thus, spectrophotometric and fluorometric analyses of the de-ribitylated compound derived by photolysis were made by comparing it with synthesized lumazines and pterins. As a result, the behavior of the de-ribitylated compound coincided perfectly with that of lumazine in spectrophotometric, fluorometric and paper chromatographic analyses, proving the de-ribitylated compound to be a lumazine. Thus, the isolated green fluorescent compound was concluded to be an 8-ribityllumazine with a lumazine molecule. Furthermore, these results show that the compound which glyoxal trapped is 4-ribitylamino-5-amino-2,6-dihydroxy-pyrimidine, the pyrimidine portion except for the glyoxal portion being on the 8-ribityllumazine molecule. The possibility that the pyrimidine is an intermediate in flavinogenesis is strongly supported by the close correlation between the inhibition of riboflavin formation and the accumulation of 8-ribityllumazine as seen in Fig. 1. The possibility is further supported by the fact that [2-14C]guanine is effectively incorporated into the 8-ribityllumazine accumulated during the inhibition of riboflavin formation.

In the present study, we isolated 4-ribitylamino-5-amino-2,6-dihydroxy-pyrimidine in the form of 8-ribityllumazine from a high flavinogenic mold, Eremothecium ashbyii and found that the pyrimidine is a possible intermediate in flavinogenesis.

REFERENCES

1) McNutt, W. S., J. Biol. Chem., 210, 511 (1954).
2) McNutt, W. S., J. Biol. Chem., 219, 365 (1956).
3) McNutt, W. S., J. Am. Chem. Soc., 83, 2303 (1961).
4) Audley, B. G. and Goodwin, T. W., Biochem. J., 84, 584 (1962).
5) Lingens, F., Oltmanns, O., and Bacher, A., Z. Naturforsch., 22b, 755 (1967).
6) Bacher, A. and Lingens, F., J. Biol. Chem., 245, 4647 (1970).
7) Kuhn, R. and Cook, A. H., Ber., 70, 761 (1937).
8) Rembold, H. and Metzger, H., Chem. Ber., 96, 1395 (1963).
9) Mitsuda, H., Suzuki, Y., and Nakajima, K., J. Nutr. Sci. Vitaminol., 19, 29 (1973).
10) Mitsuda, H. and Nakajima, K., J. Vitaminol., 18, 131 (1972).
11) Plaut, G. W. E., J. Biol. Chem., 238, 2225 (1963).
12) Goodwin, T. W. and Treble, D. H., Biochem. J., 67, 19P (1957).