The Biosynthesis of Riboflavin in *Eremothecium ashbyii*

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The ascomycete *Eremothecium ashbyii* is well known for its capability of synthesizing riboflavin. Under controlled conditions remarkably high yields are obtained. (Robinson¹, Hickey²).

The organism is usually grown under aerobic conditions (submerged) in a liquid medium containing some sources of nitrogen and carbohydrate. Synthetic media have been described, some of which support the growth excellently, and give high yields of riboflavin. (Schopfer and Guilloud³, Yaw⁴, Hickey²).

According to MacLaren⁵, there is no direct correlation between the growth of the organism and its ability of synthesizing riboflavin. It was therefore found of importance to investigate the dependence of riboflavin synthesis upon growth, and experiments have been performed where washed mycelium of *Eremothecium ashbyii* was incubated in a solution of inorganic salts.

As acetic acid enters a great many metabolic processes, the possible role of this compound in the riboflavin synthesis has been investigated.

EXPERIMENTAL AND RESULTS

Eremothecium ashbyii (American Type Culture Collection, 6747) was kept on agar slants of the composition: 4.0 % glucose, 0.5 % peptone (Difco), 0.5 % yeast extract (Difco) and 1.8 % agar. The cultures were incubated at 28° C for 24 hours, and stored at room temperature. Cultures used in the experiments were transferred daily.

The medium for the liquid cultures had the same composition, except that the agar was omitted. The flasks were shaken in a water-bath at 28° C, at a rate of approximately 100 strokes per minute.

In the experiments with washed mycelium the following buffer was used: 100 parts 0.9 % sodium chloride, 5 parts 1.15 % potassium chloride, 5 parts 3.83 % magnesium sulfate (epsomite) and 15 parts 0.1 M di-sodium hydrogen phosphate buffer of pH 7.4. The pH of the mixture was adjusted to 6.9 before use.

Growth and riboflavin production. Preliminary experiments were made by measuring the oxygen consumption of mycelium suspended in fresh medium, and in phosphate buffer, by the direct method of Warburg. Mycelium was collected by centrifugation from a 24 hours old shaking culture, washed twice with buffer and resuspended in fresh medium. The oxygen consumption per unit time increased at a rate of 6–8 % per 10 minutes, indicating abundant growth. When washed mycelium was incubated in phosphate buffer no growth took place, the volume oxygen taken up being linear with time.

Table 1. Riboflavin production in *Eremothecium Ashbyii*. Washed mycelium.

Experiment No.	pH	Riboflavin, $\mu\text{g/ml}$	
		Content	Net increase
I			
Zero time	6.9	6	—
Incubated 72 h	7.0	47	41
II			
Zero time	6.9	7	—
Incubated 45 h	—	60	53

40 ml liquid medium, inoculated from agar slant, was incubated at 28° C for 48 h. Mycelium was collected by centrifugation, washed twice and resuspended in 4 × 20 ml buffer. The numbers are averages from 3 incubated flasks. The riboflavin was determined fluorometrically.

Experiments were then made to determine the formation of riboflavin when washed mycelium was incubated in phosphate buffer. Washed mycelium from a 48 hours old shaking culture was re-suspended in buffer of twice the original volume of the medium. The suspension was measured into 100 ml flasks in portions of 20 ml, and shaken at 28° C for 45 to 72 hours. The riboflavin was determined fluorometrically, and compared to a non incubated sample of the suspension (Table 1).

The data given in Table 1, clearly demonstrate that *E. ashbyii* is able to synthesize riboflavin, even when deprived of organic materials except those residing in the cells themselves, and under conditions when no growth takes place.

Experiments with 1-C^{14} acetic acid. Liquid medium (50 ml) was inoculated from a 24 hours old shaking culture. After 24 hours shaking at 28° C 10 μcurie 1-C^{14} acetate was added and the shaking continued for another 72 hours. The yield of riboflavin was 220 $\mu\text{g/ml}$ as determined fluorometrically.

Most of the riboflavin was isolated by extraction with butanol and precipitation with petroleum-ether¹, 40 mg carrier riboflavin was added and the mixture was crystallized from water/alcohol. The riboflavin was then counted by direct plating by means of an ordinary end window G.M. counter. The activity recovered in riboflavin was very low, amounting to less than 0.01 % of the added C^{14} .

Degradation experiments. A small portion of the C^{14} containing riboflavin was degraded without addition of carrier. An aqueous solution was made alkaline with sodium hydroxide and irradiated with a 100 watt lamp for 2 hours. The impure lumiflavine was extracted by shaking the acidified solution several times with chloroform. After removal of the chloroform by distillation the residue was dissolved in 1 *N* sodium hydroxide and heated on a boiling water-bath for 30 minutes, made acid with acetic acid and again extracted with chloroform^{2,7}. After removal of the chloroform the residue should consist of the hydrolysis product of lumiflavin, and lumicrome, and of some unchanged lumiflavin^{2,8}.

The difficulties in obtaining small quantities of these substances in the pure state led to an attempt to separate them by means of paper chromatography. Water saturated *n*-butanol was used as the solvent, and the paper was Whatman No. 1. Standard reference substances were prepared according to Kuhn *et al.*⁷, and Karrer *et al.*⁸. The three substances gave well defined spots, visible by their fluorescence in ultraviolet light (Fig. 1).

For estimation of the distribution of C^{14} along the paperchromatogram, a strip 3.5 cm broad and holding all the spots was cut out of the paper. This strip was fitted into a double aluminum screen, the upper plate of which was supplied with a slit, 1.0 cm broad and 3.0 cm long. The slit was centered relative to the window of the G. M. tube, and the paper strip was moved past the slit, one cm at the time, and counted.

Fig. 1. C^{14} activity in degradation products of riboflavin, separated by means of paper chromatography. Counted along the intact paper strip, one cm at the time. Riboflavin formed by *Eremothecium ashbyii* incubated in a medium containing 1- C^{14} acetate.

In Fig. 1 the obtained C^{14} distribution curve is drawn, and the positions of the spots are indicated. As will be seen from the curve the C^{14} content of the hydrolysis product of lumiflavin is low compared to that of lumiflavin and lumicrome. The high activity on the right hand side of the paper is probably due to lower fatty acids.

The experiment was repeated with washed mycelium. Ten ml suspension was incubated with 50 μ curie 1- C^{14} acetate for 72 hours. The riboflavin was degraded directly in the centrifuged medium, and the degradation products were separated by paper chromatography as before. The results are given in Fig. 2.

DISCUSSION

The demonstration of riboflavin synthesis in washed mycelium of *Eremothecium ashbyii* incubated in a solution of inorganic salts (Table 1), proves that riboflavin is a product of the metabolism in the already formed cells. Thus, growth and riboflavin synthesis may be regarded as independent phenomena. However, growth factors will obviously stimulate the riboflavin formation, as more cells are formed when such substances are present in the medium.

MacLaren⁵ reported that xanthine and some other purines stimulated riboflavin synthesis in *Eremothecium ashbyii*, while the growth was little affected. Hickey² and Yaw⁴ found no effect of xanthine on growth and riboflavin formation in *E. ashbyii*. It is apparent from the papers of Hickey and Yaw that they tested the effect of xanthine as a growth factor in a sub-optimal medium. In accordance with MacLaren's data they found no such effect. In MacLaren's experiments xanthine was added to a medium where the organism grew well, and stimulated under such conditions the formation of riboflavin.

These aspects are discussed here because the results from the experiments with 1- C^{14} acetate indirectly support MacLaren's hypothesis of some purine-like substance as a precursor in riboflavin synthesis.

Fig. 2. C^{14} activity in degradation products of riboflavin, separated by means of paper chromatography. Counted along the intact paper strip, one cm at the time. Riboflavin formed by *Eremothecium ashbyii* incubated in a phosphate buffer containing 1- C^{14} acetate.

The C^{14} present in lumiflavin is split off by hydrolysis, yielding a practically inactive hydrolysis product (Figs. 1 and 2). Thus, C^{14} from 1- C^{14} acetic acid is present in carbon No. 2 in lumiflavin.

Riboflavin: R = ribityl-chain.
Lumiflavin: R = CH_3 -group.
Lumicrome: R = H.

Hydrolysis product of lumiflavin.

The purine nucleus.

According to Schulman, Sonne and Buchanan ¹⁰ the precursor for carbons 2 and 8 in the purine nucleus is formic acid, while acetic acid is not incorporated into the purines in pigeon liver extract. According to Krebs, Gurin and Eggleston ¹¹ the tricarboxylic acid cycle is not the mayor pathway of acetate oxidation in yeast and other microorganisms. The following pathway is tentatively suggested: Acetyl-coenzyme A, glycolyl-, glyoxalyl-, oxalyl- and formyl-coenzyme A. Thus, carboxyl labelled acetate would give rise to purines labelled in the positions 2 and 8. If a purine nucleus is formed as a precursor of riboflavin the carbon 8 will probably be split off, leaving the riboflavin nucleus labelled in position 2, in accordance with the reported data. It is therefore probable that a substance with purine structure is utilized in the synthesis of the riboflavin molecule.

SUMMARY

1. When incubated in a buffer containing only inorganic constituents *Eremothecium ashbyii* produced significant amounts of riboflavin.

2. Separation of the degradation products of riboflavin by means of paper chromatography is described.

3. C¹⁴ from carboxyl-labelled acetic acid is found in carbon No. 2 in the riboflavin nucleus, thus giving indirect evidence of a purine precursor in riboflavin synthesis in *Eremothecium ashbyii*.

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