The Utilization of the Ureide Carbon of Adenine in the Biosynthesis of Riboflavin in *Eremothecium ashbyii*

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There is considerable evidence that a critical reaction in purine synthesis is the closure of the purine ring by the addition of a "formate" group to 4-amino-5-imidazolecarboxamide ribonucleotide (1). Apparently the "formate" is added as an active intermediate, since formate as such has little effect in stimulating purine synthesis. Indeed, there is a demonstrable difference in the utilization of formate according to the position of its attachment to tetrahydrofolic acid (2). The biochemical reactions involved in 1-carbon metabolism have been widely studied. The imidazole, or carbon atom 2 of histidine, for example, can be transferred, via the tetrahydrofolic acid intermediate, to positions 2 and 8 of purine (3). Serine, as well, can supply a formyl group for purine synthesis (4). To our knowledge no one has suggested that adenine itself may act as a formate donor, although the relative lability of the imidazole carbon has long been recognized.

In the conversion of adenine to riboflavin in *Eremothecium ashbyii*, the imidazole carbon is lost (5). We have observed that under certain conditions this atom is incorporated into position 2 of riboflavin. The mechanism that we propose to account for this is as follows. Assuming that a purine precursor is an obligatory intermediate in the synthesis of riboflavin, the C^4 from position 8 of adenine, which is "lost" with the opening of the imidazole ring, is incorporated in the pyrimidine ring of a newly synthesized purine molecule, in position 2, and in that location becomes incorporated into riboflavin.

**EXPERIMENTAL PROCEDURE**

The organism used in this study was a strain of *Eremothecium ashbyii*, designated NRRL Y-1363. The culture conditions and experimental procedures have been described elsewhere (6). Equal amounts of [^{14}C] adenine were dispensed into five culture flasks, and various concentrations of carrier adenine were added. The cultures were incubated at room temperature (23-25°C) for 5 days with continuous agitation on a mechanical shaker.

At the end of the incubation period, 200 mmoles of unlabeled adenine were added to each flask to dilute any unmetabolized, labeled adenine remaining in the culture medium. The culture fluid was then filtered and poured through a column of Florisil, 60 to 100 mesh. The riboflavin remained in the first few milliliters of the Florisil, and 200 ml of water were passed through the column to wash out any unabsorbed materials. The riboflavin was eluted in a band with a 20% pyridine-5% glacial acetic acid-75% water mixture. The pyridine was extracted from the eluent with ether, and the water layer, containing the riboflavin, was evaporated to dryness. The riboflavin was then dissolved with hot water and the absorption spectrum was measured with a Beckman DU spectrophotometer. An aliquot was removed for measurement of radioactivity.

Carrier riboflavin was added to bring the total amount of the remainder to 100 mg. This was hydrolyzed for 1 hour at 60°C in 2 N NaOH (7). The products of hydrolysis are 6,7-dimethyl-2-keto-1-n-ribityl-3-carboxyl-1,2-dihydroquinoxaline and urea. The urea represents nitrogen atoms 1 and 3 and the carbon 2 from the riboflavin (Fig. 1). The alkaline hydrolysate was neutralized with HCl and buffered to pH 6.8 with 0.2 M phosphate in a final volume of 25 ml. After a preliminary period of flushing the mixture with CO₂-free nitrogen, a freshly prepared solution of 0.1 g of Squibb twice crystallized urease in 5 ml of water was added and the mixture was incubated for 1 hour at 37°C. The reaction was stopped by the addition of 5 ml of concentrated HCl. During the reaction and for one hour after the addition of the acid, nitrogen was bubbled through the reaction mixture and through a delivery tube into a 15-ml centrifuge tube containing 10 cc of a saturated solution of Ba(OH)₂. The CO₂ evolved by the hydrolysis of urea was precipitated as BaC₂O₄, which was centrifuged, washed with water and three times with absolute methanol. After the final washing, the BaC₂O₄ was centrifuged into a 1-inch-diameter planchet, dried, weighed, and the radioactivity was counted with the use of a thin window (1.4 mg per cm²) Geiger tube. The statistical error of counting was kept to a value below 2%.

**RESULTS AND DISCUSSION**

The addition of adenine to the growth medium of *E. ashbyii* resulted in an increase in the production of riboflavin (Fig. 2). It can be seen that at low concentrations of adenine the slope of the curve of riboflavin yield versus adenine concentration is greater than one; i.e. there is more than one additional millimole of riboflavin produced for every additional millimole of adenine supplied in the growth medium. At higher concentrations of adenine this effect vanishes, and at very high concentrations, riboflavin synthesis becomes independent of adenine concentration.

There are several ways to explain the greater-than-stoichiometric increase in riboflavin yield when adenine is added. One hypothesis is that the imidazole carbon, which is removed from
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Fig. 1. The alkaline hydrolysis of riboflavin to recover carbon 2 as urea.

Fig. 2. Riboflavin yield versus adenine concentration in growth medium. Triplicate flasks contained 15 ml each of growth medium with varying concentrations of adenine. The riboflavin produced after 7 days of incubation was assayed colorimetrically. The dry weight of mycelium produced in the various flasks did not vary significantly.

the adenine molecule before the adenine is incorporated into the riboflavin, might supply an "active formate" which is necessary for the completion of the pyrimidine ring of a new molecule of purine being synthesized by the mold. If this were so, each molecule of adenine used up in the synthesis of riboflavin would contribute the carbon atom essential for the endogenous synthesis of a new purine molecule, which would, in turn, be available for incorporation into an additional molecule of riboflavin.

There is a difficulty in testing this hypothesis. Exogenous adenine suppresses the endogenous synthesis of adenine. This feedback control system has been observed in other organisms (8) and it appears to be present in E. ashbyii as well. Goodwin found that the addition of adenine to the growth medium almost completely suppressed the incorporation of C14 from a labeled purine precursor (9). It is difficult to observe rate-limiting reactions in purine synthesis unless some mechanism exists for the rapid removal of the purine as it is formed. Such a mechanism is found in Eremothecium ashbyii. The enormous quantities of riboflavin synthesized represent a continuous strain on the metabolism. Purine, in particular, seems to be a limiting substrate for this process, and therefore the "pool" of purine is rapidly drained.

When labeled adenine was added to the culture medium, it was promptly incorporated into riboflavin, with very little loss of activity. Even though this organism has no nutritional requirement for purines, and growth is not enhanced by the addition of adenine, it uses exogenous sources of the purine, apparently with the suppression of synthesis de novo. However, in view of the abnormally high metabolic utilization of purines, if a very small quantity of exogenous adenine were administered, synthesis might not be completely suppressed, and incorporation of carbon 8 into position 2 could be seen.

McNutt (5), with 8-C14 adenine in a concentration of 750 μmoles per liter, reported very little incorporation of the activity into riboflavin. This was very likely due to the suppression of adenine synthesis by the relatively high concentration of exogenous adenine.

When Maley and Plaut (10) supplied 8-C14 adenine to Ashbya gossypii in a concentration only one-half that used by McNutt, they were surprised at the degree of incorporation of labeled carbon into the riboflavin (13% compared with 35% recovery of C14 when randomly labeled adenine was used).

In our experiments, a series of five different concentrations of 8 C14 adenine were used. The lowest concentration was 0.02 mM and the highest was 20 mM. It can be seen from Table I that when high concentrations of 8-C14 adenine were used, there was little or no incorporation of C14 in the riboflavin produced by the mold. When smaller quantities of 8-C14 adenine were used, greater incorporation of C14 occurred and this was almost exogenous ad
It still remains to be shown that the supply of "active formate" is actually the limiting factor in the synthesis of adenine. Although formate as such has little effect on riboflavin synthesis in *E. ashbyii*, the formate donor histidine does produce an increase (11). The stimulating effect of histidine is not additive to that of purines; when optimal amounts of adenine are supplied, the addition of histidine to the culture medium has no effect on the yield of riboflavin.

Also, when C₁⁴-labeled histidine was supplied in the culture medium, the activity was found predominantly in position 2 of riboflavin. Serine contributes its β-carbon, as formate, to position 2 of riboflavin (9). When unlabeled adenine was added, the incorporation of C₁⁴ from serine did not occur. This observation, although by no means conclusive evidence, lends support to the assumption that purine is an obligatory intermediate in riboflavin synthesis.

**SUMMARY**

1. The addition of adenine to the growth medium of *Eremothecium ashbyii* resulted in an increase of riboflavin production of greater magnitude than expected on a mole-to-mole basis.
2. The imidazole carbon atom was not incorporated in the aromatic ring of the riboflavin molecule but did appear in position 2.

REFERENCES

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