THE EFFECTS OF CERTAIN PURINES AND PYRIMIDINES UPON THE PRODUCTION OF RIBOFLAVIN BY EREMOTHECIUM ASHBYII[1]

JOHN A. MacLAREN

Department of Biology, Vanderbilt University, Nashville, Tennessee

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Eremothecium ashbyii is one of a number of organisms which produce considerable amounts of riboflavin, most of which is secreted by the cells into the medium (Guilliermond, 1935). The investigations of Schopfer (1944) and Schopfer and Guilloud (1945) resulted in the development of a synthetic medium for this organism which permitted growth and riboflavin production to occur under chemically defined conditions. Subsequent studies by Yaw (1948) have led to a modification of the original medium to include the amino acids, methionine and histidine.

With the determination of the nutritional requirements of *E. ashbyii*, it became possible to undertake studies concerning the mechanism of riboflavin biosynthesis by means of precursors which modify riboflavin yield. Such an approach was utilized by Yaw (1948), who tested certain synthetic compounds for precursor activity. However, none of the compounds tested was found to be active in the biosynthesis of riboflavin.

The present study is concerned with the purines and pyrimidines in the biosynthesis of riboflavin by *E. ashbyii*. These compounds were selected for the following reasons: (1) There are marked similarities in chemical structure among the purines, pyrimidines, and riboflavin, all of which contain a diazine ring, and (2) the purines and pyrimidines are naturally occurring compounds. It was therefore hoped that the information obtained would provide a better understanding of riboflavin biosynthesis, about which very little is known at present (Williams *et al.*, 1950).

MATERIALS AND METHODS

Several strains of *E. ashbyii* were studied in the course of this work. The strain employed in all of the experiments to be reported was one which produced high yields of riboflavin. It was obtained through the courtesy of Dr. O. G. Wegrich, Commercial Solvents Corporation, Terre Haute, Indiana and is referred to as strain 1.

This organism was maintained in culture on agar slants of a nonsynthetic medium consisting of the following components: 1.0 g peptone, 1.0 g glucose, 0.1 g yeast extract, 1.5 g agar, and distilled water to 100 ml. The pH of the medium was adjusted to 6.5 prior to autoclaving. The organism was transferred to fresh slants every 7 days to ensure viability and maintenance of capacity for riboflavin production.

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In the preparation of the inoculum, the organism was cultivated in a non-synthetic liquid medium of the following composition: 1.0 g peptone, 1.0 g glucose, 0.1 g yeast extract, and distilled water to 100 ml. The pH of the medium was adjusted to 6.5 before autoclaving. This medium was dispensed in 40 ml portions in 125-ml Erlenmeyer flasks. After an incubation period of 48 hours, 10 ml of the culture solution, containing spores and mycelial fragments, were withdrawn and centrifuged for 10 minutes. Following removal of the supernatant, the fungus material was resuspended in 5 ml of sterile distilled water and washed twice. Following washing, the spores and mycelial fragments were resuspended in 10 ml of sterile distilled water and used for inoculation. The volume of the inoculum was equal to 1 per cent of the volume of the medium to be inoculated. For example, 1 ml of inoculum was used to inoculate 100 ml of medium.

All glassware employed was thoroughly washed with a commercial detergent, rinsed repeatedly in tap water, cleaned with a solution of sulfuric acid and potassium dichromate, then rinsed repeatedly with tap water followed by several rinses with distilled water.

The synthetic medium employed throughout this project differed from that devised by Yaw (1948) only in the increase in the concentration of methionine from 2.5 to 5.0 mg per 100 ml and in alteration of the pH from 6.4 to 6.9. The composition of this medium is as follows: 40.0 μg thiamine, 4.0 mg inositol, 0.25 μg biotin, 1.0 g glucose, 0.1 g L-asparagine, 2.5 mg L-histidine, 5.0 mg DL-methionine, 0.05 g $MgSO_4 \cdot 7H_2O$, 5.54 ml M/15 Na_2HPO_4, 4.46 ml M/15 KH_2PO_4, and distilled water to 100 ml. The buffer components were adjusted to give the medium a pH of 6.9. The water used in the preparation of the medium was twice distilled. This synthetic medium is referred to as the basal medium.

Each purine or pyrimidine was tested for precursor activity at a concentration of 100 μg per ml in 500-ml Erlenmeyer flasks, each of which contained 100 ml of the basal medium. Xanthine and uracil, however, were tested further in concentrations greater than 100 μg per ml. The majority of the test chemicals were obtained from General Biochemicals, Incorporated.

The effects of the chemicals upon growth and riboflavin production of *E. ashbyii* were determined by comparison of results with a control.

Sterilization of all media and test chemicals in stock solutions was conducted by autoclaving the solutions at 15 pounds pressure for 15 minutes. In order to prevent the formation of precipitates and the destruction of amino acids on autoclaving, the constituents of the basal medium were sterilized in two groups; one group consisted of the phosphate buffer, asparagine, the additional amino acids and the vitamins, and the other group contained the glucose and magnesium sulfate. After these two solutions were sterilized and cooled, they were mixed under aseptic conditions.

A temperature of 25 C was employed for the incubation of all slants and liquid cultures destined for inocula. Because no constant temperature space was available in which to place the shaker, all the shaken cultures were incubated at room temperature, which varied within the range 20 C to 30 C during the period in which the work was performed. After inoculation with strain 1 of *E. ashbyii*, the

culture flasks were incubated on a mechanical shaker for 7 days. The reciprocating shaker operated with a stroke of 8 cm and at a rate of 80 strokes per minute. The experiments were performed in triplicate. In order to cope with fluctuations of temperature, each experiment was designed to be complete unto itself with all its culture flasks being exposed to the same conditions of temperature during the period of incubation.

Since most of the riboflavin produced by *E. ashbyii* is liberated from the cells during growth, it was not considered necessary to use an elaborate extraction procedure in order to prepare the material for riboflavin determination. Prior to

TABLE 1

The effects of adenine, guanine, xanthine, and thymine upon the growth and riboflavin production of Eremothecium ashbyii

CHEMICALS ADDED TO BASAL MEDIUM	GROWTH			RIBOFLAVIN YIELD			
	mat wt mg	t	p	Fluorometric μg/ml	t	p	Per cent increase or decrease in yield
Adenine	122 124 130	1.82	0.2–0.1	154 158 149	12.68	<0.01	+66
Guanine	119 127 125	1.59	0.2–0.1	130 115 108	3.28	0.05–0.02	+26
Xanthine	113 111 126	0.28	>0.5	120 108 125	3.88	0.02–0.01	+26
Thymine	121 121 122	1.02	0.4–0.3	101 91 115	1.11	0.4–0.3	+10
Control (none)	123 113 119			101 89 89			

riboflavin determinations, all cultures were adjusted to an acid reaction with 1.0 ml of 10 per cent HCl and autoclaved at 15 pounds pressure for 15 minutes to ensure liberation of the bound riboflavin. After cooling, the mycelium was removed from the solution by filtration, the mats were saved for determination of dry weights, and the pH of the filtrate was readjusted to about 6.5. The method of determining the mat weights consisted of removal of the mats from the culture medium by filtering through previously weighed filter papers and drying to constant weight in a desiccator over anhydrous calcium sulfate, followed by weighing. Riboflavin concentrations were determined by fluorometric technique (Johnson, 1946), using a Coleman model 12 electronic photofluorometer.

In the statistical treatment of the data in tables 1 and 2, the t test for comparison of two groups having equal numbers of individuals was used (Snedecor, 1946). In the present work, p values of 0.05 or below are considered significant.

RESULTS

The results of preliminary experiments indicated that it was necessary to incubate the organism for 7 days and to use an optimum inoculum size in order to obtain maximum riboflavin production. Maximal riboflavin production occurred within the pH range 6.8 to 7.5, and, consequently, an initial pH value of 6.9 was chosen for the experiments.

TABLE 2

The effects of hypoxanthine, xanthine, uric acid, and uracil upon the growth and riboflavin production of Eremothecium ashbyii

CHEMICALS ADDED TO BASAL MEDIUM	GROWTH			RIBOFLAVIN YIELD			
	mat wt mg	t	p	Fluorometric μg/ml	t	p	Per cent increase or decrease in yield
Hypoxanthine	133 144 133	1.12	0.4–0.3	166 178 166	2.31	0.1–0.05	+7
Xanthine	127 128 132	0.13	>0.5	190 190 216	4.29	0.02–0.01	+25
Uric acid	132 122 126	0.51	>0.5	168 173 173	5.10	<0.01	+8
Uracil	132 143 144	1.60	0.2–0.1	146 146 129	3.30	0.05–0.02	−12
Control (none)	139 122 128			158 163 158			

A number of experiments of a preliminary nature were performed with guanine, adenine, xanthine, uracil, thymine, and hypoxanthine as possible riboflavin precursors, with each included singly in the basal medium at a level of 100 μg per ml. In experiments with nonagitated cultures and with riboflavin determinations made colorimetrically, small increases in riboflavin formation, ranging up to 20 or 25 per cent, were noted with guanine, adenine, xanthine, thymine, and hypoxanthine, while a marked depression in riboflavin yield was noted in the presence of uracil. These findings were, in general, verified upon repetition in

shaken cultures. In an attempt to substantiate these findings, riboflavin determinations were performed employing the more acceptable fluorometric procedure, and all experiments were performed in triplicate.

The results of an experiment with adenine, guanine, xanthine, and thymine are presented in table 1. The p values for the mat weights of cultures grown on basal medium containing adenine, guanine, xanthine, or thymine, in comparison

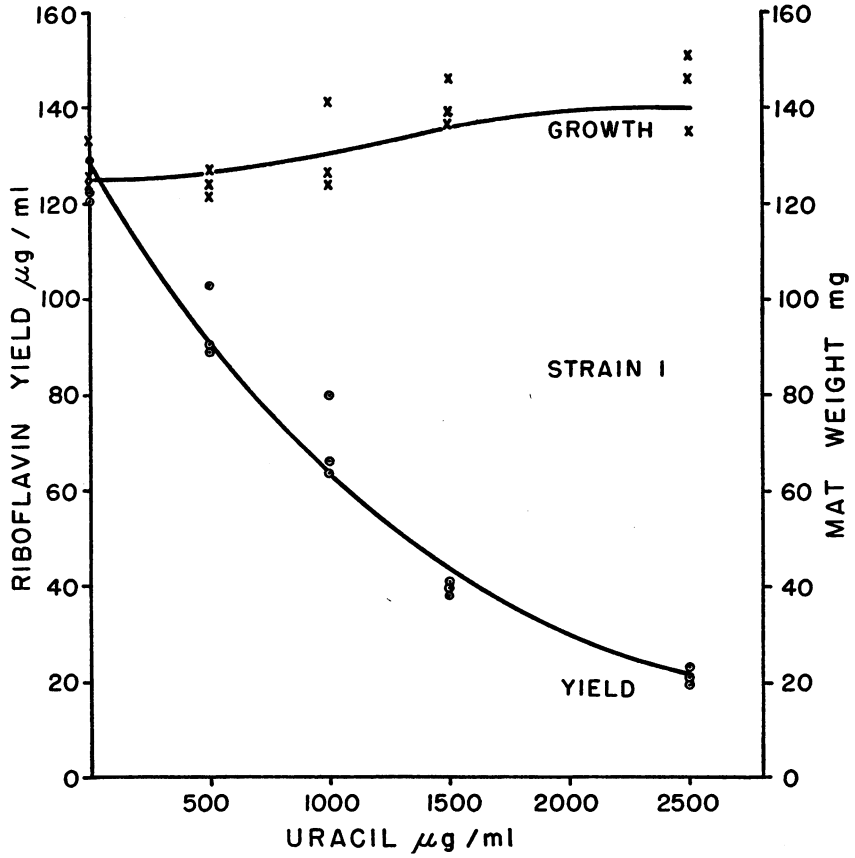

Figure 1. The effect of uracil upon the growth and riboflavin production of *Eremothecium ashbyii*.

with controls, are greater than 0.05. It may be concluded that none of these compounds, in the concentration used, had a significant effect upon the growth of strain 1 of *E. ashbyii*. Thus, any possible influence of these compounds upon the riboflavin yield is independent of effects upon the growth of the organism.

Adenine, guanine, and xanthine brought about increases in the yields of riboflavin which are statistically significant. For these compounds, the increases in yields were 66 per cent, 26 per cent, and 26 per cent, and the p values were < 0.01, 0.05–0.02, and 0.02–0.01, respectively. The 10 per cent increase in yield (p

value 0.4–0.3) obtained with thymine is not significant. In no instance was the increase in yield of riboflavin accompanied by a significant increase in the growth of the fungus.

The results of a similar experiment, in which hypoxanthine, xanthine, uric acid, and uracil were tested at the same concentration of 100 μg per ml, are presented in table 2.

The values of p (table 2) indicate that none of the test chemicals affected the amount of growth of strain 1 to a significant degree. From the values of p calculated for the riboflavin concentrations, however, it is seen that the 25 per cent increase in yield of riboflavin obtained with xanthine and the 8 per cent increase

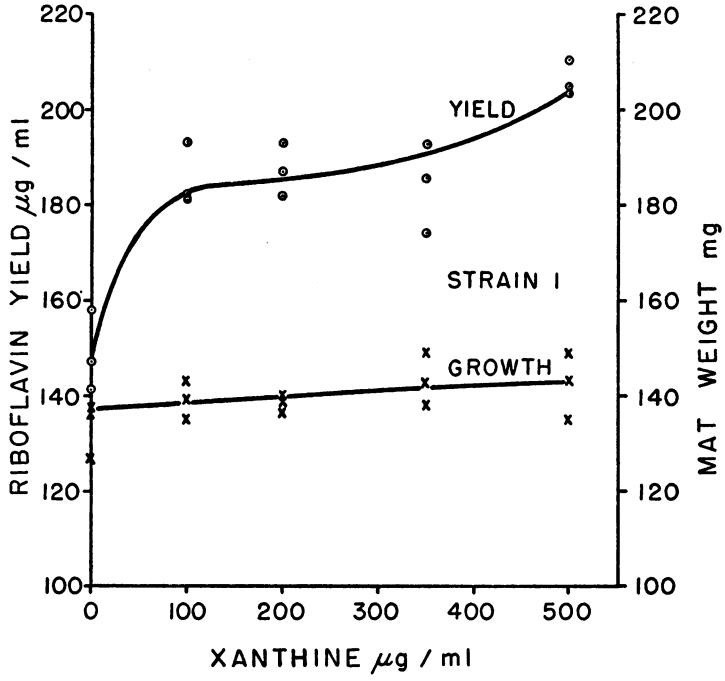

Figure 2. The effect of xanthine upon the growth and riboflavin production of *Eremothecium ashbyii*.

obtained with uric acid are significant. Although uracil did not significantly affect the growth of *E. ashbyii*, it resulted in a 12 per cent decrease in the yield of riboflavin, an amount which according to the p value is significant.

It is evident from the results of the preceding experiments, that the purines, adenine, xanthine, guanine, and uric acid are effective in promoting riboflavin production while hypoxanthine gave increases which closely approach significance. The pyrimidines, thymine and uracil, either do not significantly affect riboflavin production or else inhibit it in a significant manner.

The previous experiments were performed with an arbitrarily chosen level of purines or pyrimidines of 100 μg per ml. Because of the finding that uracil, a pyrimidine with the dicarbonyl diazine ring structure of riboflavin, has an effect upon riboflavin yield different from that of the purines, it was decided to test

uracil and the purine which it most closely resembles, namely xanthine, in a series of higher concentrations. The results with uracil are presented in figure 1 and those with xanthine in figure 2.

It may be seen from figure 1 that the inhibitory effect of uracil upon riboflavin production increases with increase in the concentration of this compound over a wide range. At the maximal concentration of uracil employed, 2,500 μg per ml, the riboflavin yield was reduced 83 per cent. At this high level of uracil, growth was actually slightly increased over the control values.

From figure 2 it may be noted that xanthine increased the riboflavin yield of strain 1 of *E. ashbyii* as much as 39 per cent without an appreciable effect upon the growth of the organism. Near maximal effects of xanthine were obtained at a concentration of 100 μg per ml, but some additional increase occurred when the xanthine concentration was raised to 500 μg per ml. At this high level of xanthine, the riboflavin yield, namely 210 μg per ml, was the highest obtained in the course of the study.

DISCUSSION

It is apparent from the data presented that a number of purines result in increasing the riboflavin yield of *E. ashbyii*. The finding that riboflavin yield is increased by the purines without accompanying increase in growth of the organism may be interpreted as strong evidence that the purines are actually precursors in the formation of riboflavin. In order for the purines to be effective in increasing riboflavin production it is required that the purine precursor must have been present in the cells of *E. ashbyii* in a limiting concentration. In other words, the ribityl side chain and the dimethylbenzene portions of riboflavin must have been produced in quantities which did not limit the riboflavin yield under the conditions employed.

In figure 3, the structural formulae of uracil and xanthine are depicted in two representations. The conventional formulae of these compounds, (2) and (3), have been rotated, as represented in (4) and (5), so that the carbonyl groups 2 and 6 of uracil and xanthine are in the same spatial relationship as the carbonyl groups, numbered 2 and 4, in riboflavin (1). The nitrogen atoms, numbered 1 and 3 in riboflavin (1), uracil (4), and xanthine (5), likewise are in the same spatial relationship.

The markedly dissimilar effects of xanthine and uracil upon the riboflavin yield of strain 1 are of interest in view of the similarity between the molecular structures of these compounds, with respect to the dicarbonyl diazine rings. Xanthine, however, has an imidazole group which is not present in uracil.

In view of the molecular similarity between the diazine rings of riboflavin and xanthine, and because xanthine promotes the riboflavin yield of strain 1 without significant effect upon the growth of the organism, it is suggested that xanthine is incorporated directly into the riboflavin molecule. Upon this assumption, N atoms 7 and 9 of xanthine (5) may become N atoms 9 and 10 of riboflavin (1). Ribose would then be linked to N atom 9 of the purine molecule. This linkage of ribose to purines by means of N atom 9 of the purines is well known as it occurs in nucleic acids. This hypothesis as to the mechanism of biosynthesis of ribo-

flavin requires that the imidazole ring of xanthine be broken in order to permit the incorporation of N atoms 9 and 7 of xanthine into the riboflavin molecule.

Uracil possesses a diazine ring and carbonyl groups like xanthine but lacks the imidazole ring. It is suggested that uracil exerts its action upon the biosynthesis of riboflavin through competitive inhibition. Its structure, then, must be quite similar to the normal substrate which it is capable of blocking. Since uracil and xanthine are quite similar, it is a reasonable possibility that uracil prevents

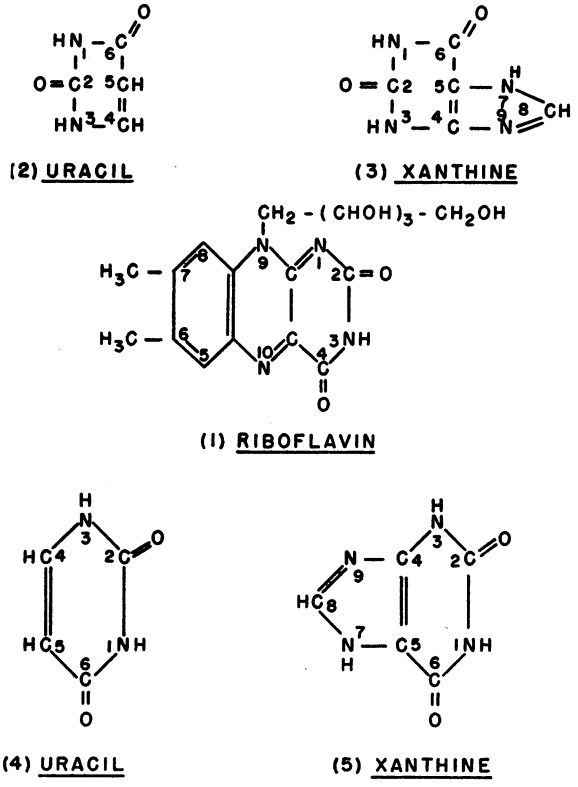

Figure 3. The structural formulae of riboflavin, xanthine, and uracil.

xanthine or some other purine, present in the cells, from participating in a normal manner in the synthesis of riboflavin.

It was suggested by Yaw (1948) that histidine is either incorporated into the riboflavin molecule or into an enzyme directly associated with the biosynthesis of riboflavin. The imidazole structure of histidine is also present in xanthine and appears to be necessary for the precursor activity of the latter compound. Thus, it is possible to reconcile this finding of Yaw (1948) with the results obtained with various purines in the present study.

When 100 μg per ml of xanthine were added to a culture, there was an increase of 36 μg per ml of riboflavin. This results in a molar conversion ratio of approximately 7:1 with respect to the number of xanthine molecules added for each additional riboflavin molecule produced.

Many of the purines tested, namely, adenine, guanine, xanthine, uric acid, and hypoxanthine, resulted in increases in the riboflavin level which were significant from a statistical point of view or closely approached significance. It is probable that these compounds are interconvertible to a particular purine which is incorporated into the riboflavin molecule. Since it is known that in other organisms both adenine and guanine may be converted to xanthine upon oxidation by appropriate enzymes (Baldwin, 1949), it would seem probable that xanthine is the compound actually incorporated into riboflavin. This hypothesis points to the possible existence of a "purine pathway" in the process of biosynthesis of riboflavin by *E. ashbyii*.

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SUMMARY

Xanthine, adenine, guanine, uric acid, and possibly hypoxanthine were effective in promoting riboflavin production in a manner independent of the growth of *Eremothicium ashbyii*. Xanthine increased the production of riboflavin as much as 39 per cent. An hypothesis is presented to account for the action of certain purines as precursors in the formation of riboflavin by *E. ashbyii* which points to the possible existence of a "purine pathway" in the process of riboflavin biosynthesis.

Uracil was found to inhibit the production of riboflavin to a marked extent, with a maximum effect of 83 per cent. It is suggested that the decrease in riboflavin yield caused by uracil may be due to competitive inhibition.

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