STUDIES ON THE SYNTHESIS OF RIBOFLAVIN BY A MUTANT YEAST, SACCHAROMYCES CEREVISIAE

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Though the synthesis of riboflavin in large amounts by microorganisms has been encountered, very little is known about the mechanism involved in the biosynthesis of this vitamin. A critical examination of the various environmental conditions for different organisms producing this vitamin showed that the optimum conditions for the production of riboflavin varied widely from organism to organism (Schopfer, 1944; Burkholder, 1943; Mayer and Rodbart, 1946). The present investigation with a mutant top yeast strain BY 2 (*Saccharomyces cerevisiae*), obtained from a bottom brewery yeast strain BY 1 (Subramaniam and Ranganathan, 1946), seemed useful to obtain, by comparison, a better knowledge of the mechanism of biosynthesis of riboflavin. Further, this organism differed from those employed in other laboratories in that it had acquired the property of excreting considerable amounts of riboflavin, whereas the parent strain was incapable of excreting any appreciable quantity of this vitamin. Purines, pyrimidines, and amino acids were tested for their effects on the biosynthesis of riboflavin since, with the determination of its nutritional requirements (Premabai, 1947) and certain environmental conditions influencing the elaboration of this vitamin (Mitra, 1950, 1952), it became possible to test these compounds for their activity. Purines and pyrimidines were selected because of their natural occurrence and their marked similarity in chemical structure to riboflavin. A similar attempt has been made recently to interpret the role of these compounds in the biosynthesis of riboflavin by *Eremothecium ashbyii* (MacLaren, 1952). Since the source of nitrogen is also an important condition for the growth as well as excretion of riboflavin by the microorganism (Burkholder, 1943), experiments were designed to test the effectiveness of supplemented sources of organic nitrogen, such as asparagine and various amino acids, in addition to the supplied inorganic ammonia.

EXPERIMENTAL METHODS

The organism was maintained on wort-agar slants. It was transferred to fresh slants every two weeks to ensure and maintain its capacity for riboflavin production. The basal medium employed for the experiments was compounded as follows: glucose, 40 g; ammonium sulfate, 2.0 g; asparagine, 2.0 g; KH_2PO_4, 0.75 g; $MgSO_4$, 0.50 g; $CaCl_2$, 0.35 g; $FeSO_4 \cdot 7H_2O$, 0.5 mg; $ZnSO_4 \cdot 7H_2O$, 0.35 mg; $CuSO_4 \cdot 5H_2O$, 0.44 mg per liter of the medium. Biotin which was an essential factor for growth and thiamin, pyridoxine, calcium pantothenate, and inositol were added also at the following levels: Biotin, 0.8 μg; thiamin, pyridoxine, and calcium pantothenate, 800 μg each; inositol, 2.0 mg per liter of the medium. The presence of iron in the medium did not affect the riboflavin production by this organism as observed in the case of *Candida guillermondia* (Levin *et al.*, 1949). The pH of the medium was adjusted to 5.0.

As it was observed that fairly good yields of riboflavin were obtained through an adequate supply of air by keeping the culture in a thin layer providing a large exposed surface for the organism to grow, without shaking, periodic shaking having a deleterious effect on growth and excretion of riboflavin, all experiments were carried out in Roux flasks (250 ml capacity) (Mitra, 1952).

Thirty ml quantities of the sterilized basal medium were dispensed into the culture flasks. The purine and pyrimidine compounds were added to give a final concentration of 50 μg per ml. Amino acids were added at 0.5 mg per ml levels. Precipitation due to the presence of salts and destruction of amino acids on autoclaving were avoided by sterilizing them separately and mixing them after cooling under aseptic conditions. The purine and pyrimidine compounds as well as the amino acids were obtained from Nutritional Biochemicals Corporation (U. S. A.).

Controlling the quantity as well as the quality of the inoculum was a very important condition for consistent results under identical conditions (Mitra, 1950, 1952). The inoculum was built up from cells cultivated in liquid medium by daily subculture for 5 to 6 days as it was found that such an inoculum was more vigorous than one from 24 hour old transfers. The cells were suspended, after centrifugation and weighing, in a known volume of the basal medium. Each flask received an inoculum at the level 0.5 mg per ml (wet weight basis).

After inoculation, the flasks were incubated in the dark for eight days at room temperature (24 to 26 C). During the first two or three days of incubation, a thick layer of growth was observed on the surface which gradually settled down, and the culture medium turned greenish yellow in color due to the formation of riboflavin. The intensity of this color increased with time till the sixth or seventh day.

Elaborate procedures for the extraction of riboflavin were not essential since most of the vitamin produced by the organism is excreted into the culture medium. Before riboflavin determination, all of the culture solutions were adjusted to an acid pH by adding one ml of 5 per cent hydrochloric acid to each one of the flasks. They were steamed for 10 to 15 minutes to ensure the liberation of the riboflavin bound to the cells. After cooling 10 ml quantities were withdrawn from the flasks and centrifuged at 3,000 rpm. Aliquots of the supernatant were used for the fluorometric determination of riboflavin by employing the method described by Kodicek and Wang (1949), with slight modifications in detail, using a Klett-model fluorometer. For determining the dry weights of the organism, 5 ml quantities were withdrawn from the culture solutions, and the cells were suspended in 5 ml distilled water after centrifuging. Turbidity readings were taken in a photoelectric colorimeter (Klett-Summerson) using a 420 mμ filter. Dry weights were calculated from a turbidity-dry weight curve. All values were calculated from the average of triplicate determinations.

RESULTS AND DISCUSSION

The effects of adenine, guanine, xanthine, hypoxanthine, thymine, uracil, and uric acid on the growth of the organism as well as riboflavin production are recorded in table 1. All the compounds tried, except uric acid, are effective in increasing the production of riboflavin without effecting an increase in the growth of the organism. This observation is in line with what has already been reported in the case of *E. ashbyii* (MacLaren, 1952), namely, that the role of these compounds in the biosynthesis of riboflavin may be in the nature of precursor activity since they only influence the production of riboflavin without a similar effect on growth. The effectiveness of adenine in increasing the riboflavin production is greater than those of other compounds at the particular level tried. However, the effects of guanine, xanthine, and hypoxanthine are also significant. The pyrimidines, thymine, and uracil also exert a similar influence on riboflavin production, though to a lesser degree. Assuming as a working hypothesis that a purine pathway exists in the biosynthesis of riboflavin, the xanthine formed upon oxidation of adenine and other compounds by appropriate enzymes, being incorporated into the riboflavin molecule, it seems probable that the organism is more capable of utilizing adenine for such a purpose than the other compounds, considering the greater effect of adenine on riboflavin production. MacLaren (1952) has observed that uracil hinders the production of riboflavin by *E. ashbyii* by exerting competitive inhibition. The presence of uracil in the culture medium is stated to prevent the normal participation of xanthine or other purines present in the cells in the synthesis of riboflavin on account of structural similarity. Our observations with the mutant yeast are

TABLE 1

Effect of purine and pyrimidines on riboflavin production and growth

ADDITIONS*	RIBOFLAVIN	DRY WEIGHT OF ORGANISMS	PER CENT INCREASE OR DECREASE IN RIBOFLAVIN PRODUCTION
	μg/ml	mg	
Adenine	72.2	29.6	+27.9
Guanine	62.1	30.2	+9.9
Xanthine	60.5	28.3	+7.1
Hypoxanthine	61.7	29.9	+9.2
Thymine	59.8	31.2	+5.8
Uracil	60.1	28.6	+6.4
Uric acid	50.3	30.0	−10.9
None	56.5	30.4	—

* 50 μg/ml basal medium.

TABLE 2

Effect of increased levels of purine and pyrimidine compounds on growth and riboflavin production

ADDITIONS	CONCENTRATION OF COMPOUNDS ADDED IN μG/ML OF MEDIUM							
	0	10	25	50	100	250	500	750
Adenine:								
Dry wt of organism in mg	28.6	30.4	32.6	30.8	29.6	30.2	29.4	30.2
Riboflavin in μg/ml	50.1	53.0	58.2	63.9	72.0	74.6	77.5	77.3
Per cent increase in riboflavin	—	+5.9	+16.1	+27.6	+43.8	+48.9	+54.7	+54.2
Guanine:								
Dry wt of organism in mg	27.5	26.8	30.5	28.3	29.0	28.4	30.0	28.0
Riboflavin in μg/ml	50.2	50.5	52.3	55.2	57.6	59.8	60.0	60.1
Per cent increase in riboflavin	—	+0.6	+4.2	+9.9	+14.7	+19.1	+19.5	+19.5
Xanthine:								
Dry wt of organism in mg	27.6	28.6	30.0	29.2	28.4	27.8	29.4	30.0
Riboflavin in μg/ml	49.3	50.9	51.6	55.1	61.7	65.1	68.7	68.2
Per cent increase in riboflavin	—	+3.2	+4.7	+11.8	+25.2	+32.1	+39.4	+38.4
Thymine:								
Dry wt of organism in mg	29.6	31.0	32.6	28.2	28.6	27.8	31.6	30.2
Riboflavin in μg/ml	52.0	52.4	54.7	55.7	57.2	59.3	59.1	59.2
Per cent increase in riboflavin	—	+0.8	+5.2	+7.1	+10.0	+14.0	+13.7	+13.8
Uracil:								
Dry wt of organism in mg	33.0	34.8	32.6	30.6	32.8	31.4	29.8	30.8
Riboflavin in μg/ml	52.3	52.4	53.2	55.5	55.9	55.5	55.6	55.7
Per cent increase in riboflavin	—	+0.2	+1.7	+6.1	+6.9	+6.1	+6.3	+6.5
Uric acid:								
Dry wt of organism in mg	32.6	31.8	30.4	29.8	31.8	32.0	30.6	29.2
Riboflavin in μg/ml	49.7	49.4	47.9	46.2	42.4	42.2	42.3	43.2
Per cent decrease in riboflavin	—	−0.6	−3.6	−7.0	−14.7	−15.1	−14.9	−13.1

different, however. Uracil, too, has a slight effect in promoting the riboflavin production while uric acid has an inhibitory effect. The growth of the organism in both cases is not affected.

All these compounds were tried also in a series of higher concentrations to find the maximum activity levels as well as any possible deviations from the observed effects at higher concentrations. The results are presented in table 2. It is clear from table 2 that for each of the compounds tested there is an optimum level beyond which it ceases to have any further effect. Adenine exerts the maximum influence on the production of riboflavin at these increased concentrations with xanthine as the next. Thymine and uracil do not appear to be of much significance in this context. The inhibitory effect of uric acid continues to increase up to a concentration of 100 μg per ml and decreases slightly at higher concentrations. In all these cases, as before, the growth of the organism is unaffected, lending further weight to the possibility of a purine pathway in riboflavin synthesis. The fact that uracil and uric acid exert different effects on riboflavin production by this organism and by *E. ashbyii* indicates the existence of different mechanisms for the elaboration of this vitamin in different organisms. However, this hypothesis requires further experimental support.

The effect of supplementing organic nitrogen in the form of amino acids is shown in figure 1. With serine and phenylalanine, riboflavin production is hindered considerably, the effect being similar on growth. The addition of tryptophan brought about a greater inhibition of both growth and vitamin production. Such effects may perhaps be due to the interference of these compounds in the normal metabolic functions of the organism as observed in the case of certain microorganisms (Maas and Davis, 1950; Beerstecher and Shive, 1947). Methionine, glycine, and arginine increase the yield of riboflavin (in the order mentioned) to a greater extent than the other amino acids tested. Methionine, glycine, and arginine were tried on the same molar basis to test their effectiveness, and the results are shown in table 3. Glycine and methionine seem

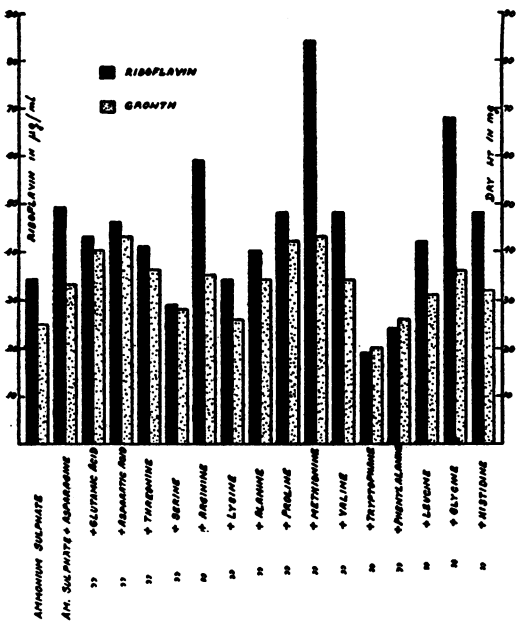

Figure 1. Effect of amino acids on riboflavin production and growth.

TABLE 3

*The effect of methionine, glycine, and arginine on riboflavin production and growth**

ADDITIONS	PERIOD OF INCUBATION IN HOURS				
	24	48	72	96	192
Methionine:					
Dry wt of organism in mg	14.6	29.6	38.3	40.1	40.5
Riboflavin in µg/ml	14.3	20.3	37.9	89.5	112.1
Glycine:					
Dry wt of organism in mg	13.2	23.7	33.9	35.1	34.8
Riboflavin in µg/ml	12.8	17.9	34.0	73.0	82.3
Arginine:					
Dry wt of organism in mg	13.1	25.6	29.8	32.1	32.8
Riboflavin in µg/ml	9.7	15.6	25.0	40.1	52.6
Control (no additions)					
Dry wt of organism in mg	12.3	23.2	25.6	26.1	26.3
Riboflavin in µg/ml	5.2	12.5	26.3	39.6	40.8

* Amino acids added at M/200 levels.

to affect the production of riboflavin favorably to a greater extent than the growth even from the earlier stages. Arginine, however, is steady in its effect on both, though to a lesser degree. The increased riboflavin production in the case of added glycine and methionine is not accompanied by a parallel increase in growth. Glycine, well known for its important role in purine metabolism, might exert, in addition to growth stimulation, considerable influence on the synthesis of riboflavin. The supplemented methionine is not used chiefly for growth, thus suggesting a secondary effect on riboflavin synthesis also. The ineffectiveness of amino acids on riboflavin production in the case of certain organisms like *Mycobacterium smegmatis* (Mayer and Rodbart, 1946) on the one hand and their favorable effect on the riboflavin production by this organism as well as some others (Burkholder, 1943) suggest the active participation of some of the amino acids in the synthesis of riboflavin in some organisms.

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SUMMARY

The effect of purine and pyrimidine compounds and amino acids on the production of riboflavin by a top yeast strain BY 2, *Saccharomyces cerevisiae*, is described. Adenine, guanine, xanthine, hypoxanthine, thymine, and uracil were effective in increasing riboflavin production while uric acid exerted an inhibitory action. They did not affect the growth. It is suggested that purines may mediate the synthesis of riboflavin by this organism. Of the amino acids tested, tryptophan, phenylalanine, and serine inhibited growth as well as riboflavin production while all the others increased both of them. Methionine and glycine had more marked effects on riboflavin production. The role of amino acids in riboflavin synthesis by the organism is discussed.

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