EFFECTS OF VARIOUS METABOLITES (SUGARS, CARBOXYLIC ACIDS AND ALCOHOLS) ON RIBOFLAVIN FORMATION IN NON-GROWING CELLS OF _ASHBYA GOSSYPII_

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Summary The effects of various sugars and sugar derivatives on flavinogenesis were examined using non-growing cells of a high flavinogenic mold, _Ashbya gossypii_. Glucose, fructose and galactose were found to be the most stimulative. Glycerol and glucono-δ-lactone were less stimulative; next in order were n-propanol, n-butanol, glycols and butanediols, which were likewise effective; acetate, lactate and pyruvate were slightly stimulative. In contrast, ribose, xylose, arabinose, ribitol, citrate, succinate, oxaloacetate, glyoxylate and malate were rather inhibitory, in additions at 1.0%. Among these compounds, ethanol (1%) greatly stimulated riboflavin formation. Maximum flavinogenesis with the above stimulants was attained by the additions of 1% ethanol, 1.25–3.0% glucose, 1.25% glycerol, 4.0–6.0% propane and butanediols, 1.0% pyruvate and 0.9% acetate after 37 hr incubation, respectively. These compounds inhibited flavinogenesis with increasing concentrations above their optimum concentrations. The stimulation effect of ethanol far exceeded those of other stimulants but ethanol had almost no effect on growth and pH values during incubation. With the addition of ethanol (1%) during incubation, maximum formation (1,776 μg/g wet mycelia) of riboflavin was achieved when added at the start of incubation and the most effective utilization was observed when added at the logarithmic phase of flavinogenesis, although the maximum formation of riboflavin in the latter case was much lower than in the former case. The relation of sugar metabolism, especially ethanol metabolism, to flavinogenesis was discussed with the flavinogenic activities of these additives.

1 The contents of this paper were partially presented at the annual meeting of The Vitamin Society of Japan at Osaka (June, 1975).

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We previously reported that guanosine triphosphate (1–3) is the most direct nucleotide precursor and is converted to riboflavin via 4-(1'-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine (4, 5). In addition, we demonstrated the clear identity of structures (6) and functions (7) in differentiating between the pyrimidine intermediate and the byproduct formed in the riboflavin synthetase reaction. While, BACHER and LINGENS (8, 9) and LOGVINENKO et al. (10) detected two pyrimidines, thought to be intermediates or their derivatives, 2,4,5-triamino-6-hydroxypyrimidine and 4-(1'-D-ribitylamino)-2,5-diamino-6-hydroxypyrimidine from the mutants of yeast. Accordingly, the biosynthetic pathway of the pyrimidine and pyrazine moieties in the isoalloxazine ring of riboflavin has been fairly well elucidated.

However, the metabolic pathways of the o-xylene ring and the ribityl side chain on the riboflavin molecule are still unclear, in spite of many works with labelled (11–18) or nonlabelled compounds (19–21).

In the present paper, we tried to obtain some knowledge on the sources of the o-xylene ring, the 4-carbon compound at the biosynthetic level of 6,7-dimethyl-8-ribityllumazine, or the ribityl side chain, using non-growing cells of a high flavinogenic mold, Ashbya gossypii.

MATERIALS AND METHODS

Materials. Sodium acetate, sodium pyruvate, sodium formate, n-propanol, iso-propanol, n-butanol, ethylether, ethylacetate (95%), formic acid (90%), acetoin dimer and ribitol were purchased from Wako Pure Chemical Industries, Ltd., Osaka. Ribose from Kohjin Co., Ltd., Tokyo. Other tested compounds were from Nakarai Chemicals, Ltd., Kyoto. Peptone from Daigo Nutritive Chemicals, Ltd., Osaka was used for the preculture and the culture in a basal medium and that from Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo was used for the slant culture. Yeast extract and sucrose were from Nakarai Chemicals, Ltd., Kyoto. Tween 80 was from Wako Pure Chemical Industries, Ltd., Kyoto. Malt extract and agar were from Difco Laboratories, Inc., Detroit, U.S.A. Potato extract was prepared from commercially available potatoes which were cut up and boiled in water (5 ml/3 g) for about half an hour. Methylglyoxal (40% in water), glyoxal (40% in water) and acetoin (80% in water) were used as the reagents under test. Diacetyl dimer was prepared by self-condensation in an alkaline solution (22). Other chemicals were of the best quality available.

Fermentation. Ashbya gossypii (IFO 1355) was obtained through the courtesy of the Institute for Fermentation, Osaka, and maintained on an agar slant containing 2.0% potato extract, 0.5% yeast extract, 2.0% sucrose and 2.0% agar (pH 5.5). The mycelia were subcultured every seven days on an agar slant consisting of 1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract and 2.0% agar (pH 6.5). A loop of the mycelia was taken from the seven days agar
slant and transplanted to a preculture medium (50 ml) in a 300 milliliter Erlenmyer flask containing 2% glucose, 1% peptone and 0.1% yeast extract (pH 6.5). After culturing for 1 day, an appropriate volume (5 ml) of the culture was transferred to a basal medium (500 ml) in a 2-liter Sakaguchi flask containing 3% glucose, 0.8% peptone, 0.2% yeast extract, 0.2% KH₂PO₄·2H₂O, 0.1% NaCl, 0.01% MgSO₄·7H₂O and 1.8% Tween 80 (pH 7.2), the combinations of which were best to promote both growth and riboflavin formation in the mold, then cultured on a reciprocal shaker (82 oscillations/min) at 27°C for 1 day. After culturing, the mycelia were harvested by filtration and washed three times with distilled water. The mycelial pad was submitted to the non-growing cell experiment.

**Non-growing cell experiment.** The washed mycelia were starved with a mild shaking (60 oscillations/min) in 0.1 M phosphate buffer (pH 6.8) at 27°C for 8 hr. After starvation, the mycelia obtained by filtration were washed and resuspended at the ratio of 1 g mycelia/20 ml buffer in the same buffer containing test compounds, which were vacuum-infiltrated into the mycelia (4). The mycelial mixture was successively incubated in the dark with a gentle shaking (82 oscillations/min) at 27°C for the indicated time. After incubation, the mixture was separated to mycelia and medium by filtration. The mycelia was heated in a constant volume of distilled water (2.5 ml/g) in the dark on a water bath at 80°C for 10 min and centrifuged. The supernatant and the medium mentioned above were used to determine the absorbance at 450 nm.

**Determination of riboflavin content, growth and pH values.** The content of riboflavin formed were calculated from their absorbances using a millimolar extinction coefficient of 11.2 at 450 nm for riboflavin and expressed as "total flavins (µg/g wet mycelia initially used)" being the sum of the respective amounts in the medium and in the mycelia containing riboflavin, FMN and FAD. Growth was determined by measuring the weight of the mycelia on a filter paper harvested by filtration right after incubation. The pH values were determined in the medium obtained through filtration right after incubation.

**RESULTS**

1. **Flavinogenic activities of various metabolites in the glycolytic pathway, pentose phosphate pathway, tricarboxylic acid pathway and 2,3-butanediol pathway**

   The effective compounds as the origins of the 4-carbon compound and the ribitol side chain were sought using non-growing cells of *A. gossypii* fed with various metabolites and their derivatives at 1%. Results are given in Table 1. Glucose, fructose and galactose being possible metabolites in a glycolytic pathway showed fair stimulation of riboflavin formation. While, the metabolites in a nonoxidative pathway of the pentose phosphate pathway, ribose, arabinose, xylose and ribitol, produced no effect or the inhibitory effects for flavinogenesis. However, glycerol and glucono-δ-lactone being possible metabolites in a oxidative
Table 1. Effects of various sugars, carboxylic acids and alcohols on riboflavin formation in non-growing cells of *Ashbya gossypii*. The mycelia (1 g) starved were suspended respectively in 0.1 M phosphate buffer (20 ml) containing the indicated compounds at 1% and incubated under the same conditions for 37 hr. After incubation, the flavin contents in the mycelia and in the medium were separately determined spectrophotometrically. The values were summed up and expressed as the relative values (%) in comparison with the control. The results in five different experiments were arranged on the basis of riboflavin formation in each control experiment (100%), which varied respectively in the mean values (μg/g wet mycelia) of 13, 16, 17, 14 and 17 among experiments.

| Substance       | %    | Substance       | %    |
|-----------------|------|-----------------|------|
| Control         | 100  | Propionate      | 33   |
| Glucose         | 295  | Butyrate        | 67   |
| Fructose        | 224  | Methanol        | 100  |
| Galactose       | 140  | Ethanol         | 1,133|
| Ribose          | 77   | n-Propanol      | 200  |
| Xylose          | 105  | iзо-Propanol    | 97   |
| Arabinose       | 82   | n-Butanol       | 152  |
| Ribitol         | 45   | sec-Butanol     | 83   |
| Pyruvate        | 138  | Benzylalcohol   | 67   |
| Acetate         | 185  | Ethylether      | 83   |
| Lactate         | 145  | Ethylacetate    | 133  |
| Glycerol        | 220  | Ethyleneglycol  | 133  |
| Thioglycerol    | 86   | Propyleneglycol | 150  |
| Glycerate       | 71   | Trimethyleneglycol | 217 |
| Glucono-δ-lactone | 243 | 1,3-Butanediol | 167  |
| Glucuronate     | 145  | 1,4-Butanediol | 167  |
| Acetoacetate    | 82   | 2,3-Butanediol | 183  |
| Citrate         | 102  | Acetoin         | 145  |
| Fumarate        | 26   | Acetoin dimer   | 75   |
| Malate          | 47   | Diacetyl        | 38   |
| Succinate       | 140  | Diacetyl dimer  | 50   |
| Oxaloacetate    | 47   | Glyoxal         | 46   |
| Glyoxylate      | 87   | Methylglyoxal   | 83   |
| Formate         | 67   |                 |      |

pathway of the pentose phosphate cycle enhanced riboflavin formation to a little less extent than hexoses as above. Monoatomic alcohols, methanol, n-, iso-propanol, n-, sec-butanol and benzyl alcohol were not stimulative except for n-propanol and n-butanol. Whereas, ethyleneglycol, propyleneglycol and trimethyleneglycol or 1,3-butanediol, 1,4-butanediol and 2,3-butanediol stimulated riboflavin formation by 1.5–2.0-fold compared to the control. On the other hand, the metabolites in a tricarboxylic acid cycle, citrate, malate, fumarate, oxaloacetate, succinate and glyoxylate inhibited riboflavin formation except for citrate without effect and succinate being a week stimulant. Carboxylic acids, formic, propionic, butylic and glyoxylic acids and acetoacetate restricted riboflavin formation as well although acetate, lactate, pyruvate and glucuronate (sodium
salts) were slightly stimulative. In addition, among the members and their derivatives in a 2,3-butanediol pathway, acetoin and 2,3-butanediol were stimulative but diacetyl, acetoin dimer and diacetyl dimer were inhibitory. The trapping agents, which are known to conjugate with the diamino- and triamino-type pyrimidine to form the pteridine ring (23-25), glyoxal, diacetyl, diacetyl dimer and methylglyoxal, reduced riboflavin formation. Among all these compounds ethanol stimulated riboflavin formation to a great extent.

2. Effects of ethanol at varied amounts on riboflavin formation during non-growing cell incubation

Effect of ethanol in a 0-2.0% concentration range on riboflavin formation was examined till 86 hr after non-growing cell incubation. Results are given in Fig. 1. The additions of ethanol at varied amounts showed the clear stimulation patterns to different extent for flavinogenesis. Riboflavin formation was enhanced with increasing concentrations to 1.0% of ethanol showing a maximum production at 1% after 86 hr incubation. The enhancement of riboflavin formation was rather reduced in its concentrations above 1.0% and was a little extent at the later stage of flavinogenesis. The yields of riboflavin in the presence of 1% ethanol were far beyond those of the control (without ethanol) for a prolonged incubation period.

3. Flavinogenic activities of ethanol and metabolites in a glycolytic pathway and in a 2,3-butanediol pathway

Effects of ethanol, glucose, pyruvate, acetate and glycerol in varied amounts

![Graph](image-url)

Fig. 1. Effects of ethanol at varied amounts on flavinogenesis during non-growing cell incubation. Each ten grams of the mycelia obtained after starvation was placed in 1-liter Erlenmyer flasks which contained ethanol at the indicated concentrations in 0.1 M phosphate buffer (200 ml). Seven flasks, differing in concentration of ethanol added, were incubated till 86 hr after vacuum infiltration. During incubation an appropriate volume (10 ml) of the mycelial mixture was sampled at the indicated incubation times from each flask and used to determine the total flavins.
on flavinogenesis were studied by incubation with non-growing cells of *A. gossypii* for 37 hr. Results are shown in Fig. 2. Maximum production appears to be respectively attained at 1.0, 1.25, 1.25, 1.0 and 0.9% in the order of ethanol, glucose, glycerol, pyruvate and acetate. Riboflavin formation was more stimulative by the additions of ethanol, glucose, glycerol, acetate and pyruvate in this order. Of these compounds, the addition of ethanol at 1% greatly enhanced riboflavin formation under experimental conditions.

Moreover, non-growing cell incubation was carried out for 37 hr in the presence of various concentrations of ethanol, glucose, pyruvate, diacetyl, acetoin and 2,3-butanediol and effects of these compounds on flavinogenesis were followed. Results are shown in Fig. 3. Ethanol was most stimulative of these compounds, showing the maximum yields of riboflavin at 1.5% in this case. 2,3-Butanediol exerted the stimulation effect in higher concentrations, in which maximum formation was attained at 5%. While, the other metabolites in a 2,3-butanediol pathway, diacetyl and acetoin, exhibited only a slight stimulative effect on flavinogenesis in

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**Fig. 2.** Flavinogenic activities of varying concentrations of ethanol and possible metabolites in a glycolytic pathway. Non-growing cell incubation was carried out for 37 hr in 0.1 M phosphate buffer containing the indicated compounds at varied amounts. The experimental procedures were the same as in Table 1.
Fig. 3. Flavinogenic activities of varying concentrations of ethanol, glucose, pyruvate and metabolites in a 2,3-butanediol pathway. Non-growing cell incubation was carried out for 37 hr in 0.1 M phosphate buffer containing the indicated compounds at varied amounts. The experimental procedures were the same as in Table 1.

lower concentrations than 2,3-butanediol. Pyruvate, another significant metabolite in a glycolytic pathway, had almost no effect on flavinogenesis as in the experiments of Table 1 and Fig. 2.

Riboflavin formations were further examined in the presence of ethanol, glucose, glycerol, pyruvate, acetate and 2,3-butanediol at their optimal concentrations, eliciting the maximum formation of riboflavin detected in the experiments of Fig. 1, during non-growing cell incubation up until 68 hr. Results are given in Fig. 4. All of the additives showed the same stimulation behaviors with increased incubation times to a varied extent, except for glucose, which was effective during early incubation. The stimulation effect of ethanol (1%) became very much noticeable at the later incubation stages, in which the yields of riboflavin amounted to 1,550 µg/g wet mycelia as total flavin accounted for 62-fold those of the control (25 µg/g wet mycelia) (without ethanol) after incubation for 68 hr. 2,3-Butanediol was the highest stimulant among these compounds except for ethanol, attaining a maximum formation (145 µg/g wet mycelia) after 61 hr. Other compounds were negligible in their yields of riboflavin over the incubation period compared to ethanol.

4. Relation of various alcohols to riboflavin formation

Effects of various alcohols at varied amounts on flavinogenesis, cell growth
and pH values in the medium were studied with non-growing cells of *A. gossypii*. Results are given in Fig. 5. They indicate that ethanol, 1,3-propanediol, 1,4-butanediol, 1,3-butanediol and 2,3-butanediol, in this order, markedly stimulated riboflavin formation although their concentrations, which gave the maximum yields of riboflavin, differed respectively as follows: 1% of ethanol, 5% of 1,3-propanediol, around 4% of 1,4-butanediol, around 6% of 1,3-butanediol and 5% of 2,3-butanediol. While methanol, *n*-, *iso*-propanol and *n*-, *sec*-butanol had almost no effect on flavinogenesis compared to the alcohols mentioned above. It is to be noted that 1,3-propanediol and butanediol derivatives become the high stimulants for flavinogenesis although their effects appeared in much higher concentrations than ethanol.

On the other hand, these additives exerted only a little effect on cell growth and pH values during incubation even with their increasing concentrations to 7%, irrespective of their different flavinogenic activities, except that *n*-butanol showed low values in cell growth and 1,2-ethanediol in pH values.

5. **Metabolic fate of ethanol in riboflavin formation**

It was found in the previous section that the addition of 1% ethanol to a non-growing cell medium with *A. gossypii* noticeably stimulates riboflavin forma-
Fig. 5. Effects of various alcohols on riboflavin formation, growth and pH values. Non-growing cell incubation proceeded for 37 hr in 0.1 M phosphate buffer containing various alcohols at the indicated concentrations. The experimental procedures were the same as in Fig. 2, except that growth and pH values in the medium were determined in the way described under the MATERIALS and METHODS. Each experiment is shown in the figure by the following symbols: • ethanol, ■ methanol, △ n-butanol, ○ sec-butanol, ◊ isopropanol, ▲ 1,2-ethanediol, ® 1,2-propanediol, × 1,3-butanediol, ○ 1,4-butanediol, ▼ 2,3-butanediol, — 1,3-propanediol.

In this section, the relation of ethanol to flavinogenesis is further examined by the addition of ethanol (1%) in the course of non-growing cell incubation. Results are given in Fig. 6, show that the earlier the addition of ethanol, the higher the maximum formation of riboflavin become. However, the utilization of ethanol after addition was most effective when ethanol was supplemented at the logarithmic phase of flavinogenesis, though the maximum yields of riboflavin were much smaller compared to those with the addition at the start of incubation. Moreover, the addition of ethanol at the stationary phase of flavinogenesis, especially even after 61 hr incubation, enhanced riboflavin formation. Accordingly, these results may demonstrate that ethanol principally serves as the carbon source and the energy source needed for the formation of riboflavin although the supposition may be easily expected also from the non-growing conditions of the mold.
Fig. 6. Flavinogenic activities of ethanol added at different stages of non-growing cell incubation. Each ten grams of the starved mycelia was placed in nine flasks of a 1 liter Erlenmyer flask containing 0.1 M phosphate buffer (200 ml). A small volume of ethanol was added to the flasks containing the mycelial suspension to give a final concentration of 1% at the incubation times shown by arrow symbols in the figure, without taking account of the increase of the volume. Incubations were respectively carried out under the same conditions throughout the experiments. Riboflavin formation in the respective experiments was followed by the same methods as in Fig. 1. The control experiments are shown by solid circles.

DISCUSSION

Many studies have been done to elucidate the origins of the o-xylene ring and the ribityl side chain on the riboflavin molecule. PLAUT showed the specific incorporation of acetate (11, 12) and the poor incorporation of pyruvate (26) into the o-xylene ring by tracer experiments, but GOODWIN and TREBLE (13) exhibited the effective incorporation of labelled acetoin, but not acetate, into riboflavin. In addition, ALI and AL-KHALIDI (14) have reported that metabolites in a pentose phosphate cycle are highly incorporated but acetate, pyruvate and acetoin are slightly incorporated into the isalloxazine ring and the ribityl side chain of riboflavin, and, recently, that a glycolytic pathway is closely related to the biosynthetic pathway of the 4-carbon compound (15). BRYN and STORMER (16) found the possibility of the direct involvement of a 2,3-butanediol pathway in the originating pathway of the 4-carbon unit. Thus, the origin of the 4-carbon compound remains unclear.
As to the origin of the ribityl side chain, Mailänder and Bacher (17) reported that the ribose moiety of guanosine added is incorporated without dilution into the ribityl side chain of riboflavin. While, Mehta et al. (18) and Maria et al. (20, 21) have demonstrated the possibility that ribitol conjugates with a purine intermediate to be converted to the ribityl side chain of riboflavin in contrast to the results obtained by Mailänder and Bacher (17). However, as to the source of the ribityl side chain, the results by Mailänder and Bacher (17) appear to be more reliable.

We tried to elucidate these problems using a non-growing cell system with Ashbya gossypii in the presence of various metabolites and their derivatives. The non-growing cell system was developed using the closely related mold to A. gossypii, Eremothecium ashbyii and the resting conditions of the mold under the experimental system were confirmed in detail (4, 27, 28). The non-growing conditions of A. gossypii can be also ascertained by comparison of cell growth and pH values before and after incubation and of them by the additions of various compounds at various concentrations, as seen in Fig. 5. In this experimental system, the members in a glycolytic pathway, glucose, fructose and galactose, were most stimulative for flavinogenesis except for ethanol. Successively, the possible metabolites in an oxidative pathway of the pentose phosphate cycle, glycerol and glucono-δ-lactone, were as stimulative, as in E. ashbyii (29). Next, alcohols, n-propanol, n-butanol, glycols and butanediols, were, likewise, stimulative. In addition, other metabolites in a glycolytic pathway, acetate, lactate and pyruvate, were the weak stimulants in flavinogenesis. While, ribose, xylose, arabinose and ribitol which are the possible metabolites in a nonoxidative pathway of the pentose phosphate pathway, rather inhibited the formation of riboflavin. This appears to agree with the results with A. gossypii by Tanner et al. (30) but is not compatible with the results with E. ashbyii obtained by Alì and Al-Khalidi (14). Almost all the members in a tricarboxylic acid cycle inhibited flavinogenesis. In the present experiment, the greatest finding is that ethanol shows a very effective stimulation in flavinogenesis.

Thus, the results may indicate that the non-growing cells of A. gossypii, under the experimental conditions used, are deficient in the metabolites in a glycolytic pathway, an oxidative pathway of the pentose phosphate cycle and a 2,3-butanediol pathway but sufficient in the metabolites in a tricarboxylic acid cycle and a non-oxidative pathway of the pentose phosphate cycle.

Ethanol produced the very specific stimulation effect for flavinogenesis being almost independent on the length of carbon chain of alcohols. Such a high flavinogenesis due to ethanol had no effect on cell growth and pH values during incubation. In contrast, ethanol did not stimulate growth or riboflavin formation at all in the culture of the mold on a basal medium. Accordingly, ethanol appears to be much more effectively utilized for the formation of riboflavin at a 1% concentration under the non-growing conditions of the mold. While, as seen in Fig. 6,
the addition of ethanol at the start of incubation provided the highest yields of riboflavin compared to the additions at other incubation stages. The results may indicate that the cells under experimental conditions need not only the building blocks of riboflavin but also energy and other metabolites required to maintain the non-growing conditions of the mycelia in order to form large amounts of riboflavin. It thus follows that ethanol added at the beginning of incubation flows into a glycolytic pathway under deficient, leading to “glyconeogenesis,” in the non-growing cells which are starved in the nutrient-free phosphate buffer; the cells are fulfilled with sugar metabolites and energy sources by exogenous ethanol, having no effect on growth. Such a cell condition seems to utilize, much more effectively, the remaining ethanol for the active formation of riboflavin at the logarithmic phase of flavinogenesis so as to form a large amount of riboflavin. In fact, the utilization of ethanol at the logarithmic phase of flavinogenesis was most effective as illustrated in Fig. 6.

Accordingly, ethanol may play a significant role in the elucidation of the origins of the 4-carbon unit and the ribityl side chain by tracing the metabolic fate of ethanol in the mycelia because ethanol itself is not considered to be directly utilized to form riboflavin.

On the other hand, it is to be noted that other alcohols, glycols, propane and butanediols show a good stimulation in flavinogenesis in higher concentrations. Thus, the mycelia appear to effectively utilize also these alcohols except ethanol as carbon sources and thus supply their metabolites for the formation of riboflavin.

Experiments to clarify the origins of the 4-carbon compound and the ribityl group on the riboflavin molecule are under way with these effective alcohols.

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