Development of Specific Experimental Systems for Flavinogenesis Using Non-Growing Cell of *Eremothecium ashbyii*

HISATERU MITSUDA AND KENJI NAKAJIMA

Laboratory of Nutritional Chemistry, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto (Post No. 606)

(Received June 1, 1972)

Experimental systems using non-growing cell of *Eremothecium ashbyii* to elucidate the mechanism of flavinogenesis were established in this paper.

1. It was found that vacuum infiltration of purines for 2, 3 and 5 min and moderate shaking of non-growing cell brought about a good reproducibility of the stimulatory effects on flavinogenesis by purines and furthermore, a higher flavinogenesis.

2. The addition of an inhibitor of protein synthesis, chloramphenicol, to the basal medium at the concentration of 6 mM after 1 day of cultivation resulted in a 45.1% inhibition of the growth measured after 2 days of cultivation and a 65.1% inhibition of riboflavin production measured after 4 days of cultivation. On the other hand, the addition of an inhibitor of purine de novo synthesis, sulfanilamide at 4 mM resulted in a 24.3% inhibition of the growth after 2 days and a 44.1% inhibition of riboflavin formation after 4 days respectively.

3. The addition of these drugs and another inhibitor of protein synthesis, cycloheximide to the non-growing cell medium brought about little effects on flavinogenesis and only cycloheximide showed a 25% inhibition of riboflavin production at 7 mM.

4. It was elucidated from above results that non-growing cell under such the special conditions shows little or no synthesis of protein and purine derivatives but there occurs flavinogenesis to a fair extent.

5. It was found that xanthine is the most flavinogenic purine among various purines during these experiments.

Investigations concerning the biosynthesis of riboflavin have so far depended exclusively on *in vivo* experiments. However, the problem raised by *in vivo* experiments is that the precursor of riboflavin is identical with that of DNA and RNA at the level of purine base. Thus, the contribution of purines to DNA and RNA biosynthesis must be taken into account, in addition to the contribution to riboflavin formation. This has made it more difficult to elucidate the riboflavin biosynthetic pathway in growing cell.

In addition, another problem is that the interconversions between purines, nucleosides

---

1 The contents of this paper were partially presented at the 199th Vitamin B Research Committee (October 1970).
2 溝田久輝，中島譲二
and nucleotides are markedly rapid in growing cell. Thus, the riboflavin biosynthetic pathway in which purines are directly converted into riboflavin molecule except for C(8) (1-5), has been hard to clarify even when the isotopically labelled purines are used.

Accordingly, in this paper, the authors have attempted to develop a system whereby the biosynthesis of riboflavin can be followed without interference from other metabolic pathways in the cell.

**EXPERIMENTAL**

**Materials:** Sulfanilamide, chloramphenicol, cycloheximide and purines were purchased from Nakarai Chemicals, Ltd., Kyoto.

**Method:** A fragment of flavinogenic *Eremothecium ashbyii* on 7 day agar slant was transferred into the precultural medium and cultured at 27° for 1 day on a rotary shaker. Thereafter, 5 ml of the medium was inoculated into the 500 ml basal medium of a 2 liter Erlenmeyer flask consisting of 2% glucose, 1% peptone, 0.3% yeast extract and salts, and thereafter cultured for 1 day at 27° with vigorous shaking. The mycelial pad obtained at logarithmic phase, after washing, was suspended in 0.1M phosphate buffer (pH 6.8) containing 1% glucose at the ratio of 1 g mycelia per 20 ml medium and then was starved by mild shaking at 27° for 8 hours. The mycelia obtained after starvation were suspended again in the above buffer medium containing test compounds and vacuum infiltration of test compounds into mycelia was carried out for 2, 3 and 5 min at intervals of 2 min respectively in a desiccator. Thereafter, non-growing cell incubation was done under moderate shaking for 18 hours at 27° if not otherwise stated.

The details about fermentational medium and determinations of total flavins and pH values were described in the previous paper (6).

**RESULTS**

1. **Effects of Vacuum Infiltration and Moderate Shaking on Riboflavin Formation of Non-growing Cell**

Effects of vacuum infiltration and shaking on riboflavin production of non-growing cells were examined in the presence of purines as shown in Fig. 1. These results indicate that neither shaking nor vacuum infiltration alone stimulated riboflavin formation. Furthermore, in this case, the effects of the addition of purines were difficult to reproduce, for example, as seen for hypoxanthine and xanthine. However, the two treatments together brought about a higher riboflavin production and above all a high reproducibility of stimulatory effects on flavinogenesis of various purines. In this case, riboflavin formation was accelerated in the order of xanthine, guanine, hypoxanthine and adenine, although the last, of great interest, rather inhibited riboflavin formation as compared to the control.

Furthermore, the effect of vacuum infiltration of test compounds on the riboflavin biosynthesis was examined. The methods were the

![Fig. 1 Effects of vacuum infiltration and shaking on flavinogenesis by non-growing cell of *E. ashbyii*](image)

Vacuum infiltration was done for 2, 3 and 5 min in desiccator in presence of purines (1 mM). The mycelia treated were thereafter incubated at 27° for 18 hr with moderate shaking, which was softly carried out by reciprocal shaker. At this time, above figure had "shaking" and under figure had "vacuum" treatments.

TABLE 1

Effect of vacuum infiltration of purines on flavinogenesis by non-growing cell of E. ashbyii

<table>
<thead>
<tr>
<th>Addition</th>
<th>Vacuum infiltration (a)</th>
<th>Incubation for 18 hr (b)</th>
<th>Sum (a + b)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mM)</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Adenine</td>
<td>6.0</td>
<td>7.9</td>
<td>77.3</td>
</tr>
<tr>
<td>Guanine</td>
<td>3.1</td>
<td>6.0</td>
<td>86.3</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>7.7</td>
<td>6.0</td>
<td>88.2</td>
</tr>
<tr>
<td>Xanthine</td>
<td>3.6</td>
<td>4.6</td>
<td>90.0</td>
</tr>
</tbody>
</table>

Total flavins (µg/ml)

<table>
<thead>
<tr>
<th>Addition</th>
<th>Total flavins</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>73.8</td>
</tr>
<tr>
<td>Adenine</td>
<td>76.2</td>
</tr>
<tr>
<td>Guanine</td>
<td>83.3</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>89.4</td>
</tr>
<tr>
<td>Xanthine</td>
<td>93.6</td>
</tr>
</tbody>
</table>

(a) Non-growing cell medium containing 1g mycelia and test compounds in 20ml medium (pH 6.8) was treated with vacuum infiltration for 2, 3 and 5 min and the absorbancies at 450 mÅ of filtrate after immediate filtration were determined photometrically for calculation of total flavins. (b) The mycelia accompanied by enough washing after vacuum infiltration were resuspended in non-growing cell medium containing no test compounds and were incubated at 27° for 18 hr in the dark.

*The values of columns (a) and (b) were combined.

It can be seen from Table 1 that the treatment of vacuum infiltration brings about the leakage of riboflavin accumulated in the cells and this phenomenon is more marked at the higher concentration of purines in medium, as seen in the left column of Table 1. This fact appears to reflect indirectly the physical infiltration of exogenous purines into the cells.

Furthermore, adenine always caused the most marked inclination of leakage. This may have a close relation to the inhibition of flavinogenesis by the adenine addition as seen in Fig. 1.

Moreover, as seen in the central column, vacuum infiltration of purines for only short periods brought about a good stimulation of flavinogenesis during non-growing cell incubation for 18 hr. The stimulatory effects of various purines on flavinogenesis were also in these experiments observed in the decreasing order of xanthine, guanine, hypoxanthine and adenine.

Accordingly, these data clearly indicate that vacuum infiltration results in higher flavinogenesis by virtue of physical penetration of exogenous purines into the cells.

The right column shows the combined values of those shown in the left and central column of Table 1. The stimulatory effects of purines in this column coincided well with those of the central column.

2. Effects of Chloramphenicol and Sulfanilamide on Riboflavin Formation and Growth of Growing Cell

It has been well known that chloramphenicol (7-9) is a specific inhibitor of the protein biosynthesis and sulfanilamide (10-12) is an inhibitor of purine de novo synthesis. In this section, effects of these inhibitors on growth and riboflavin formation were examined in growing cells as preliminary experiments in order to elucidate the physiological conditions of non-growing cells.

As seen in Fig. 2, chloramphenicol and sulfanilamide at the concentrations of 3, 6 mM and 2, 4 mM respectively were added to 1 day fermentation medium, because the mycelia used for non-growing cell experiment were obtained by filtering the medium after 24 hours of cultivation (thereafter called “at 1 day”).

It can be seen from Fig. 2 that pH curves show the same directions in spite of the addition of these drugs.

On the other hand, riboflavin formation was restricted by these drugs, especially by chloramphenicol. At 4 days when the stationary phase of riboflavin formation was almost achieved, riboflavin yields were inhibited over the range of 44.1 and 46.6 % by the addition of 4 mM and 2 mM of sulfanilamide and 50.2 and 65.2 % by 3 mM and 6 mM of chloramphenicol respectively.

Furthermore, effects of these drugs on growth were shown in Fig. 3. These data show that the addition of sulfanilamide and chloramphenicol at the same concentration as the above experiments results in a growth inhibition of 24.3, 32.8 and 45.1 % (the same extent at both concentration of chloramphenicol)
FIG. 2 Effects of sulfanilamide and chloramphenicol on the riboflavin synthesis of E. ashbyii on a basal medium

Cultivation was done by rotary shaker at 27°C in the dark on a basal medium (pH 5.5) consisting of 2% glucose, 1% peptone, 0.3% yeast extract, 0.2% KH₂PO₄, 0.1% NaCl and 0.01% MgSO₄·7H₂O. Inhibitors were added at 1 day of culture as shown in the figure. Cont.; control.

FIG. 3 Effects of sulfanilamide and chloramphenicol on growth of E. ashbyii on a basal medium

The methods of cultivation were the same as those of Fig. 2.

respectively at 2 days when the growth maximum is usually achieved.

3. Effects of Chloramphenicol, Cycloheximide and Sulfanilamide on Riboflavin Production of Non-growing Cell

Effects of chloramphenicol and sulfanilamide on riboflavin production and growth of a flavinogenic strain of E. ashbyii were examined in the previous section, in which chloramphenicol and sulfanilamide both were found to have a potent inhibitory effect on growth and riboflavin formation over the concentrations indicated in Fig. 2 and 3.

Now, additional effects of these drugs and another specific inhibitor of protein synthesis, cycloheximide (13-15), on riboflavin formation were followed in non-growing cells of the same microorganism. The results were shown in Fig. 4. In this experiment, cycloheximide was added in twice the concentration at which the growth of a cycloheximide-resistant yeast began to be inhibited. The addition of cycloheximide did not inhibit riboflavin formation.
not affect riboflavin formation with its increase up to 2.2 mM except for a point of 0.4 mM, but thereafter, it gradually inhibited riboflavin production, followed by a plateau in the 5-7 mM region. This inhibitory effect was observed to a small extent even at high concentration of this drug.

On the other hand, sulfanilamide rather slowly increased riboflavin yields with its increase and at 4 mM, which extensively inhibited riboflavin synthesis and growth in growing cell, riboflavin formation was increased by 6.7%. Furthermore, chloramphenicol showed a plateau region for 0 to 3 mM and then scarcely inhibited riboflavin formation with its increase. In this case, the inhibition rate was 13.3% at 6 mM which restricted quite noticeably the growth and riboflavin formation of growing cells.

As a whole, riboflavin production curves exhibited plateaus at high concentration regions of each of these drugs.

**DISCUSSION**

Vacuum infiltration has so far been applied only for plants (16, 17), not for microorganism. As shown in this paper, the application of this treatment for flavinogenic *E. ashbyii* brought about a great success in rising the yields of riboflavin and also in obtaining a high reproducibility of the stimulatory effects by exogenous purines on flavinogenesis (Fig. 1 and Table 1). It is conceivable that these effects are caused by a physical stimulation of membrane transport by compounds like exogenous purines and endogenous products, (riboflavin etc.).

Accordingly, the selection of exogenous purines by the membrane of this mold appears to be overcome to a fair extent by this treatment. The specific response of flavinogenesis to different exogenous purines can thus be examined.

Furthermore, it seems that moderate shaking results in higher yields of riboflavin by accelerating the respiration of this mold to some extent.

Accordingly, vacuum infiltration and moderate shaking were both necessary for non-growing cell experiments.

Next, physiological conditions of non-growing cells under the above conditions were pursued using various inhibitors.

First, the effects on growing cell by inhibitors, chloramphenicol (7-9) and sulfanilamide (10-12), were examined. Chloramphenicol and sulfanilamide scarcely affected the pH values even when added in high concentration. However, these drugs resulted in a noticeable inhibitory effects on riboflavin formation. The effect was especially strong with chloramphenicol added in high concentration (6 mM).

Furthermore, the effects of these drugs on growth were examined and an inhibitory pattern similar to that which was observed for riboflavin formation, was detected. The effect of chloramphenicol on growth appears to be consistent with the results of Brown (18) which were observed on the static culture of the same mold in natural nutrient broth.

As a whole, it is known that chloramphenicol is a more potent inhibitor than sulfanilamide concerning inhibitory effects on growth and riboflavin formation. However, the difference between them appears to be attributed to their different modes of action. Chloramphenicol, an inhibitor of protein synthesis, was added to 1 day old cultural medium, in which protein synthesis of this mycelia was supposed to be exponentially synthesized because the growth maximum is achieved at 2 or 3 days. For this reason, chloramphenicol should have a potent action on this mycelia.

On the other hand, the effect of sulfanilamide is speculated as follows: The pool size of nucleotides reaches a maximum at 1 day, which means that the most active synthesis of nucleotide is already finished by 1 day. Accordingly, the effect of sulfanilamide is thought to be less active when this drug was added at 1 day. Thus, a more active effect of this drug would be observed when it was added to the fermentation medium earlier during the first day of the culture.

Next, the effects on riboflavin production of these drugs and another inhibitor of protein synthesis, cycloheximide (13-15) were examined.
on non-growing cell. Cycloheximide and chloramphenicol brought about a slight inhibition but sulfanilamide rather stimulated riboflavin production at its highest concentration (Fig. 4).

These results indicate that, in non-growing cell, there are little or no inhibition at the biosynthesis level of enzymes participating in the riboflavin formation and also that there is no inhibition at the level of substrates of riboflavin synthesis conducted from purine de novo synthesis. Accordingly, it was assumed that non-growing cell appears to synthesize little or no protein and purine derivatives but synthesize riboflavin during the incubation. Thus, this system may be able to be called a coordinated enzyme system concerning the riboflavin biosynthesis.

Now, since purines de novo synthesis does not function in non-growing cells, this raises the problem of what the large amount of substrates for flavinogenesis is derived from. Although there is not an exact answer to this problem at present, these substrates seem to be supplemented from RNA and DNA, especially RNA (19-23), because riboflavin formation is initiated at the same time as autolysis in this mycelium. As the data which support this consideration were obtained partially in the next paper, further discussion of this problem will be done later.

REFERENCES