Stimulatory Effects of Purines on Flavinogenesis by 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)

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Purine metabolism in relation to the riboflavin biosynthesis was examined using non-growing cell of *Eremothecium ashbyii*.

1. It was found that xanthine is the most effective purine for flavinogenesis among various purines tested in the stimulation experiments using non-growing cell.

2. The UV light absorption spectra of purines in non-growing cell medium were determined during the incubation. The conversions of adenine to hypoxanthine at the earlier stages and hypoxanthine to xanthine at the later stages were detected. Furthermore, the conversion of adenine to hypoxanthine was found to be located endogenously.

3. The membrane transportable effects of purines were examined by photo-metrically determining the residual purines in the medium during the incubation and thus noticeable results which commonly have a unique plateau regions were obtained. However, any marked differences between added purines were not observed.

4. Fluctuation of purine pools in cell was followed, using cation exchange resin, Dowex-50 × 4 in the presence of various purines. Thus, guanine was found to be accumulative but adenine was not detected in cell. Moreover, xanthine did not convert to any other purines and guanine also was not convertible except for its conversion to xanthine.

5. Dynamic changes of purines in the 24 hr incubation medium were followed by the use of cation exchanger. However, the accumulation of particular purine derivatives was not observed.

6. Only the third nucleotide fraction in the water eluting fraction of the column chromatography by cation exchanger characteristically fluctuated with the incubation. This fraction was found to consist of adenosine monophosphate and guanosine monophosphate. The changes of these nucleotides seem to be closely related to flavinogenesis.

7. Xanthine contents inside and outside the cells at 24 hr of incubation were determined by using Florisil column and Dowex-50 column. Thus, guanine appeared to be converted to xanthine at the later stages of the incubation. Other purines were also well in accordance with the results of Summary 2.
It has been well established that purine is directly incorporated into the riboflavin molecule after rupture of the imidazole ring accompanied by elimination of C(8) (1-5). But, it has been obscure which purine is the most immediate precursor of riboflavin.

However, the cause of this problem seems substantially attributable to the activity of the salvage pathways of exogenous purines varying with bacterial species and cell conditions.

Thus, the present work was undertaken to elucidate the relationship between salvage pathways of exogenous purines and riboflavin biosynthetic pathway in the flavinogenic strain of *Eremothecium ashbyii*, using a 'non-growing cell system which was established in the previous paper (6).

**EXPERIMENTAL**

*Materials and Methods:* Dowex 50W×4 resin (100-200 mesh) which was used to separate acid soluble purines and nucleosides in *Eremothecium ashbyii* was purchased from Nakarai, Co. Ltd. and transferred to H⁺ form with 1N NaOH and 3N HCl. This resin was packed into a column (8×1cm) and washed with distilled water before use.

The samples applied to this column were prepared by extracting mycelia (1g) with a mixture of H₂O (2ml) and 3M HClO₄ (0.4ml) and neutralizing the extract to pH 7.0 with 3M KOH under the cold (4°C). Ten ml of the clear supernatant obtained were loaded on to this column, followed by the solvent I: H₂O (120 ml) with the flow rate of 6ml/5 min and the solvent II: 1N HCl (200ml) with the flow rate of 3ml/5 min, which in the said order flowed into the mixing chamber containing 200ml water. The second solvent eluted all the known purines and nucleosides.

Furthermore, the separation of nucleotides accumulated in the cells by the addition of guanine and xanthine was carried out by the column chromatography of Dowex 1×2 (formate, 0.9×30 cm) with a step-wise gradient elution system (7).

The absorption values of eluate from these columns were followed automatically at 253.7 mμ by Uvicord having 0.3 cm light path. Ultraviolet light absorption spectra of added purines during non-growing cell incubation were obtained by Shimazu multi-purpose automatic photometer MPS-50L type.

Moreover, purified Florisil was purchased from Nakarai, Co. Ltd. and was packed into a column (4×1.5 cm) in order to separate xanthine and fluorescent compounds, especially riboflavin. At this time, xanthine was eluted with distilled water from the column, while flavins were adsorbed on the upper layers of the column. From this eluate, xanthine contents were directly determined by using 7.5 as molar extinction coefficient at 260mμ after the adjustment to pH 7.0.

For the identification of purines, nucleosides and riboflavin by paper chromatography, the following solvent systems were used: Solvent I: n-butanol-ethanol-2N ammonia water (50:2:5) (8), Solvent II: 1M ammonium acetate buffer (pH 3.8)-ethanol (3:7.5) (9), Solvent III: n-butanol-acetic acid-water (4:1:1) (10), Solvent IV: iso-propanol-2N ammonia water (7:3) (11, 12), Solvent V: 0.1M phosphate buffer (pH 6.8)-ammonium sulfate-n-propanol (100ml:60g:2ml) (13) and Solvent VI: collidine saturated by water (14).

Further identification of purines, nucleosides and riboflavin was carried out by means of UV light absorption spectra and eluting position on a column chromatogram with reference compounds.

Methods of non-growing cell incubation and procedures of various determinations were described in the previous papers (6, 15).

**RESULTS**

1. Effects of Exogenous Purines on Riboflavin Production of Non-growing Cell

It was reported (16) that xanthine and guanine are the most flavinogenic purines among various purines in growing cell. Accordingly, it was suggested that there probably exists two pathways, guanine-xanthine pathway and adenine pathway to the riboflavin biosyn-
BIOSYNTHESIS OF RIBOFLAVIN IN E. ASHBYII

Fig. 1 Effects of purines on riboflavin production by non-growing cell of E. ashbyii

Vacuum infiltration was carried out in presence of various purines (1 mM) and thereafter the media were incubated at 27°C in the dark. A constant volume of the mixtures was withdrawn from non-growing cell media at the indicated times and after filtration, total flavin was determined.

However, as exogenous purines also are incorporated into DNA and RNA in growing cell, it is equivocal that added purines indicate the flavinogenic effects through any pathways in the cells. On the other hand, it is quite possible as seen in the previous paper (6) that exogenous purines do not contribute to DNA and RNA synthesis in non-growing cell. Thus, exogenous purines added in non-growing cell medium should provide more direct contribution to the biosynthesis of riboflavin.

As seen in Fig. 1, the addition of purines to non-growing cell medium, being different from the case of growing cells, brought about the simple and parallel curves of riboflavin production with the incubation time. These results indicate that riboflavin formation proceeds through a constant and stable pathway in non-growing cell. Furthermore, it can be clearly seen from this figure that the most active stimulant for flavinogenesis is xanthine during all phases of this incubation, followed by guanine, hypoxanthine and adenine. In this connection, it is interesting to note that adenine has been observed to inhibit riboflavin formation partially in E. ashbyii grown on a completely defined medium (16) and a semi-defined medium containing peptone (17).

2. Conversion of Purines in the Incubation Medium of Non-growing Cell

Although it was found that the most stimulatory purine is xanthine also in the non-growing cell experiment, this raised the problem of whether xanthine exhibits the high flavinogenesis because it is converted most rapidly to other more flavinogenic purine derivatives, or it passes most effectively through the membrane of this mycelia.

In this section, then, by determining successively the UV light absorption spectrum of different exogenous purines in non-growing cell incubations, it was examined whether purines added to the medium were transferred to other purine derivatives.

Fig. 2 indicates that, after the addition of adenine, changes of the absorption maximum of the medium were detected gradually for 0-3 hours after the incubation and markedly for

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Fig. 2 Ultraviolet light absorption spectra of the Media containing various purines during non-growing cell incubation

The concentration of various purines at 0 hr was 0.5 mM respectively. The same sampling methods as Fig. 1 were used for determination of each spectrum.
3-6 hours as shown by the shift of the maximum absorption point from 260 to 250 mμ. These changes probably indicate the conversion of adenine to hypoxanthine because the latter has a characteristic absorption maximum at 250 mμ. Thereafter, the absorption values at 250 mμ continued to decrease up till 12 hours without showing any changes of the spectrum. With guanine, its spectrum indicated no changes from 0 hr to 12 hr but the absorption values decreased continuously with its characteristic absorption curve which has two absorption maxima of 250 and 280 mμ. With hypoxanthine, the values were gradually reduced up till 12 hr but at 24 hr, the conversion to a xanthine-like compound was observed, indicated by the absorption maximum at 270 mμ. Furthermore, with xanthine, the absorption curve continued to diminish up till 12 hr, showing a similar development to that of guanine.

Accordingly, it can be seen from this figure that the conversions of adenine to a hypoxanthine-like compound at 6 hr and of hypoxanthine to a xanthine-like compound at 24 hr are detected, while guanine and xanthine show no changes at the various incubation times.

Next, the permeability of each purine was examined by reploting respectively the absorption values at 260 mμ in adenine, at 280 mμ in guanine, at 250 mμ in hypoxanthine and at 270 mμ in xanthine, taking their values at 0 hr as 100 % on the ordinate and the incubation time on the abscissa.

Fig. 3 demonstrates that all purines added at 0.5 mM were absorbed rapidly up till 3 hr and thereafter had plateau regions up till 6 hr without exception; the plateau of hypoxanthine lasted even longer (3-9 hr.). This more marked pattern, of great interest, was observed in guanine and xanthine, especially in the latter. After these plateaus, they were rapidly absorbed again up till 12 hr, at which time not xanthine but guanine indicated the most marked decrease in relative absorption. However, after 12 hr, second moderate plateaus were observed in all purines. At 24 hr, guanine had completely been absorbed in spite of the fact that other purines were partially left in the medium. At this time, the permeability curve of adenine should follow that of hypoxanthine after 6 hr, because adenine seems to have been fully converted to hypoxanthine at 6 hr.

In conclusion, there were no marked differences in their permeabilities during the incubation, although interesting absorption curves of added purines were commonly obtained.

Accordingly, the conclusion appears to be that the stimulatory effect of xanthine on flavinogenesis was attributed neither to the effectiveness of permeability nor probably to the conversion to other specific stimulatory purine derivatives because of its lack of changes in the medium.

3. The Conversion of Adenine to Hypoxanthine and of Hypoxanthine to Xanthine during Non-growing Cell Incubation

Experiments were done to elucidate the conversion of adenine to hypoxanthine and hypoxanthine to xanthine as detected.

The medium obtained at 12 hr after the adenine addition at 0.5 mM was adsorbed on charcoal and the residues after elution and evaporation were dissolved in 10 ml water. This solution thereafter was loaded on to the column of Dowex-50 W (H+) and separated by a step-
BIOSYNTHESIS OF RIBOFLAVIN IN E. ASHYII

Fig. 4 Dowex-50W × 4 (H⁺) column chromatography of non-growing cell medium at 12 hr containing adenine (5×10⁻⁴ M) and spectroscopies of hypoxanthine fraction

Non-growing cell medium which contained 0.5 mM adenine at 0 hr was treated with charcoal at 12 hr incubation and the thus obtained sample was applied to the cation exchanger accompanied by step-wise gradient elution using 1N hydrochloric acid. For this chromatography see the details in Methods. H: hypoxanthine, B₂: riboflavin.

wise gradient elution systems described in detail in the Experimental Section. The data are recorded in Fig. 4, which indicates that there was a large peak only in the hypoxanthine fraction but no peaks in the eluting position of adenine. Furthermore, UV light absorption spectra of the hypoxanthine fraction showed the characteristic pattern of hypoxanthine in 0.1N HCl and 0.1N NaOH as shown in the right, above Fig. 4.

On the other hand, paper chromatography of the hypoxanthine fraction was done using five solvent systems and their RF values well were found to correspond to those of authentic compound. Moreover, the eluting position of this fraction from Dowex-50 column also was coincident with that of authentic sample.

Accordingly, it was shown definitively that adenine begins to be converted to hypoxanthine after incubation of non-growing cells for 3 hr.

Next, it was examined by the method described below whether this conversion was carried out inside or outside the cells.

Non-growing cell medium containing 0.5 mM adenine was divided into two parts at 3 hr after the start of the incubation. One, containing no mycelia, was obtained by filtration of the medium through filter paper, and another was kept without filtration. Both media were continuously incubated under the same conditions for 2 further hours. During this incubation, UV light absorption spectra of their supernatant obtained by withdrawing a part of their mixtures at 0.5, 1 and 2 hr respectively were determined. The results obtained indicated that no changes were detected in the filtered medium, in spite of the fact that spectral changes of adenine to hypoxanthine were observed in the medium containing mycelia.

The same experiments as those done at 3 hr were carried out also at 4.5 hr after the incubation but no conversions of adenine to hypoxanthine were observed in the medium not containing mycelia.

Accordingly, it is concluded that exogenous adenine is not converted to hypoxanthine in the medium by an enzyme excreted outside the cells but that exogenous adenine enters into the cells and is then excreted outside the cells as hypoxanthine formed in the cells.

Next, to elucidate the conversion of hypoxanthine to xanthine, experiments similar to the above ones were carried out, except that Florisil column was used before the Dowex-50 column treatment.

At 24 hr after the addition of hypoxanthine at 0.5 mM, at which time the conversion of hypoxanthine to xanthine appears to be active, the non-growing cell medium was run through the Florisil column after charcoal treatment. In this column chromatography, purine derivatives were eluted by water but flavins and other fluorescent compounds were adsorbed on the upper layer of the column. Thus, the fraction eluted by water contained no flavins. Furthermore, thus obtained purine fraction was concentrated and applied to the Dowex-50 column, followed by the elution of water and 1N HCl.

Thus, the peak detected in the water eluting fraction was identified to be xanthine by (I) the characteristic UV light absorption spectra as seen in the upper right corner of Fig. 5, (II) the eluting position on the chromatogram and (III) paper chromatographies using various solvent systems with authentic compound.
Non-growing cell medium which contained 0.5 mM hypoxanthine at 0 hr was treated with charcoal at 24 hr incubation and the thus obtained samples were applied to a Florisil column for the removal of fluorescent compounds, especially riboflavin. Purine fraction from this column was treated again with charcoal and thus concentrated samples were run through Dowex-50W × 4-(H+) column. The methods of the Dowex-50 column chromatography were the same as those of Fig. 5.

Accordingly, it is evident that the two peaks which were found at 24 hr by the addition of hypoxanthine are xanthine and hypoxanthine, appearing in this order on the chromatogram. This result indicates that the conversion of hypoxanthine to xanthine is only taking place after 12 hours of incubation of the non-growing cells, because no changes of the spectrum were detected up till 12 hr in the case of hypoxanthine addition of Fig. 2. But, even at 24 hr some of the added hypoxanthine had not been converted into xanthine.

4. Effects of Exogenous Purines on the Endogenous Purine Pools during Non-growing Cell Incubation

In this section, effects of exogenous purines on the endogenous purine pools were examined by column chromatography of Dowex-50W, to which the acid soluble extract of flavinogenic mycelia incubated in the presence of various purines was applied.

Fig. 6 shows the typical patterns of purine and nucleoside pools at 0, 12 and 24 hr after non-growing cell incubation. These patterns contained nucleotides, riboflavin and xanthine in water eluting fraction and hypoxanthine, adenosine, thymine and adenine in 1 N hydrochloric acid eluting fraction in this order.

It can be seen that the addition of adenine results in an increase of the hypoxanthine pool, whose size is quite similar to that caused by addition of hypoxanthine at 12 hr incubation. Above all, it is very interesting that an intracellular adenine fraction is not observed at all on this chromatogram even by the addition of high concentration of adenine to the medium.

The increase of the hypoxanthine pool caused by the supplementation of adenine is thought to agree well with the results found in
Furthermore, at 24 hr incubation, the fluctuation of only the adenosine pool was observed by the addition of adenine and hypoxanthine.

On the other hand, riboflavin production indicated 43.7% inhibition by the adenine addition and 11.3% stimulation by the hypoxanthine addition against the values of the control at 24 hr incubation.

Next, effects of guanine and xanthine on the endogenous purine pools were examined using the above method.

As you can see from Fig. 7, purine and nucleoside pools were detectable but very small during the incubation. However, the addition of guanine brought about a large accumulation of endogenous guanine at 12 hr, while the addition of xanthine gave quite similar elution patterns to those of the control, except the B2X fraction, being the mixtures of riboflavin and xanthine.

These results show that exogenous xanthine is not converted to any other purines after the incorporation into the cells.

Furthermore, at 24 hr incubation, the same patterns as those obtained at 12 hr were observed, although there was a slight decrease of their pools.

It was found from above results that the addition of guanine brings about a large guanine pool in the cells. This fact means that the guanine phosphoribosyltransferase (EC 2.4.2.8) which catalyzed the conversion of guanine to guanosine monophosphate, is rigidly regulated by feedback inhibition by the product.

In this experiment, riboflavin production was stimulated 26.3% and 27.6% by the addition of guanine and xanthine respectively at 24 hr of the incubation.

5. Fluctuation of Exogenous Purines in Non-growing Cell Medium at 24 hr of Incubation

It was further examined in non-growing cell medium at 24 hr using the Dowex-50 column whether added purines were converted into other purines in the medium, or whether any kinds of purines were excreted out of the cells.

The elution systems of this column are identical with those of the previous sections. The media at 24 hr incubation were adsorbed on charcoal, eluted and evaporated. The samples thus obtained were diluted to 10 ml with water. This solution was loaded on to the Dowex-50 column and eluted with water, followed by 1N hydrochloric acid.

It can be seen from Fig. 8 that after addition of guanine, xanthine and adenine, there were no other fractions except hypoxanthine if the B2X fraction was neglected. However, the amount of accumulation of hypoxanthine after the addition of adenine was large.

On the other hand, after the addition of hypoxanthine, a large peak of hypoxanthine itself and an accumulation of guanine and adenosine were observed, although the reason for the accumulation of guanine and adenosine were unknown. These facts coincided
well with the measurements obtained inside and outside the cells during non-growing cell incubation.


Nucleotides were eluted with water as the unadsorbate with riboflavin and xanthine in this column system. However, they were differentiated in three fractions on the chromatogram as seen in Fig. 9. It is interesting to note that the third nucleotide fraction, which is exhibited by black shadow in Fig. 9, fluctuates noticeably with the addition of purines, especially guanine and xanthine, in spite of the fact that the first and second nucleotide fractions scarcely fluctuate during the whole incubation period.

The first curve of Fig. 9 indicates that 1 day cultural mycelium contains a large amount of the third nucleotides. But, by starving the cells for 8 hr before the incubation, the third nucleotide pool begins to decrease and, at 6 hr, the pool of them is minimal. Thereafter, on the contrary, the pool begins to increase and shows the maximum values again at 12 hr.

This shrinkage stage of the pool is coincident with the first plateau region of purine absorption curve over 3-6 hr. Furthermore, the stage which is showing the maximum of the third nucleotide fraction corresponds to the second rapid absorption stage of purine absorption curve up till 12 hr. Accordingly, these correspondence may demonstrate that, after 6 hr, the endogenous purine pools which were filled up to their maximum size begin to be exhausted for synthesis of nucleotides through the salvage pathway of purines via xanthine. Thus exogenous purines enter again rapidly into cell to complement the endogenous purine pools.

Another pathway to supply the endogenous purine pools is thought to be the degradative pathway of DNA and RNA, especially RNA, because the third nucleotide fraction of control at 12 hr also increases in spite of the fact that the de novo synthesis of purine nucleotides is not involved in this system. Accordingly, it is possible to consider that a large amount of precursors of flavinogenesis is supplied from RNA under normal conditions.

![Fig. 8 Dowex-50 W×4-(H+) chromatographies of non-growing cell media at 24 hr incubation in presence of various purines (5×10^(-4)M)](image)

Non-growing cell media at 24 hr containing various purines were applied to a Dowex-50 column followed by the methods of Fig. 4. Solvent I: H2O, Solvent II: 1 N HCl. B2: riboflavin, X: xanthine, H: hypoxanthine, G: guanine, AdR: adenosine.

![Fig. 9 Fluctuation of endogenous nucleotide pools on Dowex-50 W×4-(H+) chromatograms during non-growing cell incubation in presence of guanine and xanthine (5×10^(-4)M)](image)

Endogenous nucleotide pools in water eluting fraction of the Dowex-50 column chromatography (Fig. 7) were pursued with the incubation time in the addition of guanine and xanthine. Fluctuated fraction of nucleotides consisting of only adenosine monophosphate and guanosine monophosphate was shown by "black shadow" in the figure. For this experiment see the details in Experimental. Starv.: starvation, Cont.: control, G: guanine, X: xanthine, B2: riboflavin. Solvent: H2O. 
Furthermore, it is greatly noticeable that, by the addition of purines, especially xanthine, the third fraction increases to a great extent even compared to the addition of guanine.

Further, at 24 hr when riboflavin production almost reaches the stationary phase, the third fraction decreases again to a trace.

These results indicate that the third fraction accumulated at 12 hr when riboflavin begins to be produced exponentially, thereafter may be completely funnelled into riboflavin formation because purine and nucleoside pools in cell and medium demonstrate no expansion at 24 hr of incubation (Fig. 3 and 7).

Accordingly, these data suggest that the riboflavin biosynthetic pathway proceeds through neither purine base nor nucleoside type but nucleotide type.

Furthermore, at this time, it is conceivable that exogenous xanthine is convertible to nucleotide and thus a large amount of the accumulated nucleotide may be converted to riboflavin. As a result, the higher yields of riboflavin appear to be brought about by the addition of xanthine.

Next, it is quite interesting to know what the third nucleotide fraction consists of. To elucidate this problem, anion exchanger (Dowex-1×2) was used here (7). Then a more purified third nucleotide fraction to be loaded on to Dowex 1×2 column was obtained by using a Dowex-50 system of modified column size and flow rate of water since the fraction was not completely separated from other neighboring peaks as seen in Fig. 9. The separation by Dowex 1×2 column chromatography was carried out by the solvent systems of formic acid-ammonium formate.

Thus, the third nucleotide fraction was found to contain AMP and GMP.

Accordingly, the nucleotide monophosphates like AMP and GMP appear to fluctuate most markedly during non-growing cell incubation and contribute to the riboflavin biosynthetic pathway.

7. Determination of Xanthine Content inside and outside Cell at 24 hr of Non-growing Cell Incubation in the Presence of Various Purines

Although the stimulatory effects of various purines on flavinogenesis have been explained to be exerted by conversion to the more flavino-genic xanthine after the addition of them (4, 21), xanthine has not been clearly detected so far inside and outside the cell of E. ashbyii. Accordingly, experimenters in this field explained the above result as follows: Although other purines are converted to xanthine, it is promptly utilized for riboflavin formation and thus its existence is not detected.

However, the existence of xanthine was definitely detected in the non-growing cell experiments described above. Accordingly, this fact strongly supports the above suggestion concerning the purine metabolism in relation to the riboflavin biosynthesis.

In this section, more detailed determinations of xanthine inside and outside cells were done as described below at 24 hr of non-growing cell incubation. The amounts of xanthine were calculated from the water eluting fraction obtained from Florisil column, on which B2 and xanthine fraction eluted from the column of Dowex-50 were separated.

As you can see in Table 1, the addition of hypoxanthine caused more accumulation of xanthine in “cell+medium” than the addition of adenine did. After the addition of xanthine or guanine, xanthine was found to have accumulated in “medium” at concentrations quite similar to

| Table 1 |
| Xanthine content* in non-growing cells and medium incubated at 27°C for 24 hours |
| Cells | Medium | Cells + Medium |
| µmoles | µmoles | µmoles |
| Control | 0.53 | 0.22 | 0.75 |
| Adenine** | 0.28 | 1.35 | 1.63 |
| Hypoxanthine | 1.22 | 1.77 | 2.99 |
| Control | 0.22 | 2.85 | 3.17 |
| Guanine | 0.28 | 10.97 | 11.25 |
| Xanthine | 0.22 | 11.14 | 11.36 |

Riboflavin and xanthine fraction on Dowex-50 column chromatograms of non-growing cell and medium incubated for 24 hr was loaded on to a Florisil column to remove riboflavin and then, xanthine content was directly determined from the xanthine fraction eluted by water after adjusting the pH values to 7.0.

* The content was calculated from 7.5 at pH 7.0 as molar extinction coefficient of xanthine.

** Purines were added at 5×10^-4 M.
those of "cell + medium".

These data indicate that the conversions of adenine to hypoxanthine and hypoxanthine to xanthine occur as well as the conversion of guanine to xanthine, and that the xanthine thus formed appears to be promptly excreted into the medium at the later stages of the incubation. Moreover, it can also be seen from Table 1 that these large amounts of xanthine in "medium" are not easily utilized even after 12 hr of the exponential phase of riboflavin production. From this consideration, it is suggested that xanthine may not be directly converted to the pyrimidine derivative which is the immediate precursor of riboflavin. Thus, the formed and accumulated compounds from xanthine, probably being nucleotides, may be transferred to a pyrimidine derivative.

**DISCUSSION**

It has been well established that purine is directly converted into the riboflavin molecule via the pyrimidine derivative after elimination of C₈ (1-5). However, adenine, guanine or xanthine have all been reported as the most stimulatory purine on riboflavin formation (1, 18-20).

The experiment in this paper were performed in order to find the order of stimulating activity of the purines on riboflavin formation using the experimental system of non-growing cell of *E. ashbyii* as developed in the previous paper (6).

It was found that xanthine, structurally the molecule most closely related to riboflavin, is also the most stimulatory purine in non-growing cell experiments. Then, the problem raised whether this effect can be attributed to its membrane transport activity or to its conversion to other flavinogenic purine derivatives. However, the results obtained indicate that the effect is not a result of the effectiveness of membrane transport.

On the other hand, in experiments that further followed the stimulatory effects of added purines, it was found that exogenous adenine is converted to hypoxanthine at earlier stages of flavinogenesis and at later stages to xanthine, while guanine is converted to xanthine at later stages of flavinogenesis. Thus, the stimulating effects of these purines on riboflavin production can be respectively explained by their conversion to the most flavinogenic purine, xanthine, as suggested by Brown et al. (21). Furthermore, at this time, it may be assumed that these purines bring about an expansion of nucleotide pool through the salvage pathway via xanthine, thus funnelling an increased nucleotide pool into the riboflavin biosynthetic pathway.

Another point of interest is that exogenous purines rapidly entered into the endogenous purine pools which were exhausted by starvation of the cells. However, the endogenous purine pool at this stage appeared not to be used immediately for the synthesis of nucleotide because the first plateau regions were observed promptly after 3 hr, which may indicate the maximum of the endogenous purine pools. Moreover, these results may indicate that endogenous purines are difficult to convert to nucleotides even when present at high concentrations in the medium until corresponding nucleotides are exhausted to a certain extent. However, after 6 hr when nucleotide synthesis rate seems likely to be high, the endogenous purine pools appear to be exhausted through the salvage pathway via xanthine and thus exogenous purine is thought to be noticeably absorbed again into cell to complement the endogenous purine pools. After 12 hr, thus accumulated nucleotide pools, especially consisting of AMP and GMP, appear to be utilized for the exponential riboflavin synthesis.

You can see from above considerations that there seems to exist a special ingenious regulation mechanism between purine pools and nucleotide pools. At this time, one can expect quite possibly that their regulation system is a feed-back inhibition by the nucleotides related to respective purines as pointed out by Berlin et al. (22).

Furthermore, as a whole, the purine pool has until now been considered markedly expandable and independent of nucleotide pool (23, 24). But the results obtained in these experiments indicate that the endogenous purine pools appear to have a constant size as particularly
The width of arrow mark shows the rapidity of the stream. Wide black arrows indicate the direction of purine interconversions in non-growing cell of E. ashbyii which was elucidated in this paper. Riboflavin biosynthetic pathway was demonstrated via GMP mainly through the salvage pathway of xanthine. A: adenine, H: hypoxanthine, X: xanthine, G: guanine, B2: riboflavin, AMP, IMP, XMP and GMP: adenosine, inosine, xanthosine and guanosine monophosphates.

seen in the guanine pool of Fig. 6. On the other hand, it was found that nucleotide pools like AMP pool and GMP pool are markedly affected by exogenous purines.

Under these conditions, the riboflavin biosynthesis is carried out at a constant rate, according to a sigmoidal curve, without the stepwise behavior detected in the exogenous purine absorption curve. On the other hand, from the above results, purine pools and nucleotide pools with their flexible nature seem to extend to their own maximum sizes in the course of incubation. Accordingly, it is suggested that there may be some unexpected regulation at some stages before and after the rupture of imidazole ring of the nucleotide if the riboflavin biosynthesis proceeds through one of the nucleotides. However, this problem may not be resolved untill the riboflavin biosynthetic pathway is elucidated in more details in future.

In conclusion, riboflavin biosynthetic pathways from various purines to riboflavin as pictured in Fig. 10 were based on the results obtained in this paper.

REFERENCES