EFFECTS OF 8-AZAGUANINE ON RIBOFLAVIN PRODUCTION AND ON THE NUCLEOTIDE POOLS IN NON-GROWING CELLS OF EREMOThECIUM ASHBYII

Hisateru Mitsuda and Kenji Nakajima

Laboratory of Nutritional Chemistry, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606, Japan

(Received May 4, 1973)

The effects of a guanine analogue, 8-azaguanine, on the riboflavin biosynthesis and the nucleotide pools were examined using non-growing cell systems of flavinogenic and non-flavinogenic strains of E. ashbyii.

1) 8-Azaguanine, as such, strongly inhibited riboflavin formation at low concentrations. The halfway inhibition point was detected at $5 \times 10^{-5}$ M.

2) The inhibition of riboflavin biosynthesis by 8-azaguanine was completely overcome by the supplementation of purines. Xanthine gave the most efficient recovery from this inhibition.

3) Fluctuation of the nucleotide pools in non-growing cells of flavinogenic and non-flavinogenic strains were examined using Dowex 1 × 2 (formate) in the presence of 8-azaguanine 18 hr after start of incubation. As a result, five new peaks appeared on the chromatogram in the flavinogenic strain whilst only one new peak was observed in non-flavinogenic strain. Furthermore, with the addition of xanthine with 8-azaguanine, peaks I, II and IV disappeared from the column, peak III was unchanged and peak V decreased to a fair extent.

4) Five peaks in flavinogenic strain were identified to be (I) uridine, (II) 8-azaguanine, (III) guanosine, (IV) 8-azaguanosine and (V) 8-azaguanosine triphosphate in the order of elution from the column and one peak in non-flavinogenic strain was found to be 8-azaguanine.

It has been reported many times that guanine is the most immediate pre-

---

1 The contents of this paper were partially presented at the 199th Vitamin B Research Committee (October 1970) and at the Annual Meeting of The Vitamin Society of Japan at Hiroshima (October, 1970).

2 湯田久輝，中島謙二
cursor of riboflavin among various purines (1–3). The authors also found a strong stimulatory effect of guanine as shown in previous papers (4–6).

Furthermore, it has been established that other pteridine derivatives and folic acid are formed from GTP through the imidazole ring opening reaction accompanied by elimination of C(8) (7–12). Experiments with labelled purine (13–15) showed that C(8) of purine base is discharged after the opening of the imidazole ring also in the biosynthetic pathway of riboflavin.

Accordingly, it is possible that an analogue of guanine may be a specific inhibitor by acting as the precursor analogue of riboflavin, especially, in non-growing cells of E. ashbyii.

In this paper, a guanine analogue, 8-azaguanine, which is different from guanine only in that the analogue has a nitrogen atom in place of C(8) of guanine, was added to the non-growing cell medium, and the effects on riboflavin production and on endogenous nucleotide pools were examined.

EXPERIMENTAL

Materials. 8-Azaguanine, other purines and their ribosides were purchased from Sigma Chemical Company. Other chemicals were described in a previous paper (16).

Methods. Anion exchange column chromatography (Dowex 1 × 2, formate) was used with the same methods as those described in a previous paper (16).

Fractions accumulated in cells by the addition of 8-azaguanine were concentrated by charcoal treatment. The fractions were identified using the following methods: 1) Paper chromatography, 2) Ultraviolet light absorption spectra in acid and alkaline solution, 3) From the eluting position on the column chromatogram, and 4) Determination of pentose and phosphate of the ribotide as described below. Identifications of base moieties of unknown ribosides and ribotide were also carried out by 1, 2 and 3 after acid hydrolysis in 1 N hydrochloric acid (16). Experiments with 1, 2 and 3 were always done with corresponding known compounds as references.

The determination of ribose and phosphate of the compounds accumulated by the addition of 8-azaguanine was done by the method of DISCHE (17) after acid hydrolysis in 1 N hydrochloric acid for ribose and by the method of AMES and DUBIN (18) for phosphate except that 0.5 N sulfuric acid was added in place of 1 N sulfuric acid promptly after the acid hydrolysis of the nucleotides by 2 N hydrochloric acid for 1 hr at 120°C.

Paper chromatography was done by an ascending method at room temperature using the following solvents: (1) 1 M acetate buffer (pH 3.8)-ethanol (3: 7.5) (19), (2) butanol-acetic acid-water (4: 1: 1) (20), (3) 0.1 M phosphate buffer (pH 6.8)-ammonium sulphate-n-propanol (100 ml: 60 g: 2 ml) (21) and (4) butanol-ethanol-2 N ammonia water (50: 2: 5) (22).
Purines, ribosides and ribotides on the chromatograms were detected under an ultraviolet lamp. Riboflavin was detected under another lamp emitting a longer wavelength.

The UV absorption spectra of the eluted compounds were determined automatically over wavelengths of 210 m\(\mu\) to 370 m\(\mu\) in 0.1 N hydrochloric acid and 0.1 N sodium hydroxide.

Non-growing cell experiments were carried out as described previously (23).

RESULTS

1. Effect of 8-azaguanine on riboflavin production during non-growing cell incubation

Non-growing cells were incubated at 27°C for 18 hr in the presence of 8-azaguanine at the concentrations indicated in Fig. 1 under conditions described previously (23). As seen in Fig. 1, riboflavin formation by non-growing cells is markedly inhibited by low concentrations of the inhibitor. The half way inhibition point was observed at 5 \(\times\) 10\(^{-5}\) M of 8-azaguanine. However, activity was promptly recovered upon the addition of various purines as seen in Fig. 2. At this time, especially, xanthine at 5 \(\times\) 10\(^{-4}\) M resulted in a complete recovery of inhibition.

![Graph](image.png)

Fig. 1. Effect of 8-azaguanine on riboflavin production by non-growing cells of *E. ashbyii*. Non-growing cell incubation was done at 27°C for 18 hr after vacuum infiltration of 8-azaguanine at various concentrations. For the experimental conditions of non-growing cell incubation see the details in a previous paper (23)
2. Utilization of 8-azaguanine by non-growing cells of *E. ashbyii*

Utilization of 8-azaguanine was followed continuously by determining changes in UV absorption spectra of the drug when it was added to the non-growing cell medium at $5 \times 10^{-4}$ M concentration.

It can be seen from Fig. 3 that 8-azaguanine in the medium is gradually absorbed into cells with incubation time. Furthermore, it seems possible that the conversion of 8-azaguanine to 8-azaxanthine is not involved because no changes in absorption pattern were observed at 270 m$\mu$. This may indicate that 8-azaguanine is absorbed into this mycelium as such from 0 to 12 hr of incubation. This result is the same as that obtained after guanine addition (6).

On the other hand, an increase of absorbanc$y$ at 250 m$\mu$ was observed during the 3–9 hr incubation. This phenomenon possibly demonstrates the leakage from this mycelium of an unknown compound having an absorption maximum at 250 m$\mu$. This compound seems to be hypoxanthine because among the well
known natural purines hypoxanthine has an absorption maximum at 250 m\(\mu\). However, this fact will have to be decided after more details are obtained in future.

Fig. 3. Ultraviolet absorption spectra of 8-azaguanine with non-growing cell incubation. An appropriate volume of non-growing cell incubation medium was withdrawn at the indicated times and each spectrum was automatically determined after the 20-fold dilution of the medium. The methods were the same as those in a previous paper (6).

Next, the utilization of 8-azaguanine was examined by observing the percentage of absorption values at 290 m\(\mu\) for various times against those at 0 hr. This wavelength was selected to avoid the influence of other factors. Furthermore, the correction for influence of riboflavin to respective values at 290 m\(\mu\) was done using the absorption ratios of 290 m\(\mu\) to 450 m\(\mu\).

Figure 4 shows that the concentration of 8-azaguanine decreases very slowly in comparison with that of guanine although both exhibit similar curves which have a plateau region around 3–6 hr. This means that mycelium has a noticeable, specific selection for 8-azaguanine. However, riboflavin formation was strongly inhibited by this drug in spite of such a low permeability. Accordingly, 8-azaguanine appears to be a potent inhibitor of riboflavin formation.

3. Acid-soluble nucleotide pools in non-growing cells of flavinogenic or non-flavino- genic strains in the presence of 8-azaguanine

An anion exchanger, Dowex 1 \(\times\) 2 (formate), was used to examine some unknown intermediate or precursor of riboflavin which may have accumulated in the cells when riboflavin production was noticeably restricted by the addition of 8-azaguanine. The acid-soluble extract used in the chromatography was prepared by the perchloric acid procedure as described previously (16) from the non-growing cells incubated for 18 hr at 27°C.

As seen in Fig. 5, the same pattern as that obtained in growing cells was observed in the control. As this pattern shows a large amount of nucleotide triphosphate, one may assume that the autolysis of the cells was not yet initiated after 18 hr of non-growing cell incubation. However, the pattern demonstrates
The results of Fig. 3 were replotted taking corrections for the contribution of riboflavin to the absorption spectra.

The addition of 8-azaguanine at $5 \times 10^{-4}$ M brought about the appearances of five new peaks as shown in this figure although riboflavin production were inhibited 33\% (in the second pattern of Fig. 5). However, further addition of xanthine at $10^{-3}$ M completely reversed the inhibition of the riboflavin formation. At this time, peaks I, II and IV, in the order which they were eluted from column, disappeared and peak V diminished; only peak III remained constant (in the third pattern).

Furthermore, addition of only xanthine brought about no characteristic changes in the pattern except that the nucleotides were present in smaller amounts (in the fourth pattern). But riboflavin formation was accelerated 43\% upon xanthine addition when compared to the control.

On the other hand, in the non-flavinogenic strain, it can be seen from Fig. 6 that only one peak corresponding to the second peak of flavinogenic strain was accumulated by the addition of 8-azaguanine. This is quite different from the appearances of flavinogenic strain. At this time, riboflavin production was inhibited 41\%.
Fig. 5. Effect of 8-azaguanine on the nucleotide pool in non-growing cells of flavinogenic strains. 8-Azaguanine and xanthine were added at $5 \times 10^{-4} \text{M}$ and $10^{-3} \text{M}$, respectively. The non-growing cells were incubated for 18 hr. The methods were the same as those described in previous paper (16). For the identification of various compounds see the details in Experimental.
Fig. 6. Effect of 8-azaguanine on the nucleotide pool in non-growing cells of non-flavinogenic strain. Experimental conditions were the same as those of Fig. 5.
4. Fluctuation of adenosine nucleotide pools in non-growing cells of flavinogenic or non-flavinogenic strains

The relative amounts of adenosine nucleotide pools on column chromatograms of Fig. 5 and 6 were recorded in Table 1.

| Addition | Flavinogenic Strain | Non-flavinogenic Strain |
|----------|---------------------|-------------------------|
|          | None | 8-Azaguanine | 8-Azaguanine + Xanthine | Xanthine | None | 8-Azaguanine |
|          | (μmoles) | (μmoles) | (μmoles) | (μmoles) | (μmoles) | (μmoles) |
| AMP      | 0.07 | 0.07 | 0.06 | 0.15 | 0.71 | 1 |
| ADP      | 0.40 | 0.20 | 0.34 | 0.36 | 0.54 | 0.30 | 0.4 |
| ATP      | 1.99 | 2.9 | 1.62 | 1.12 | 7.5 | 0.12 | 0.25 | 0.4 |
| AMP+ADP | 2.46 | 0.16 | 2.02 | 1.63 | 1.96 | 0.12 | 0.25 | 0.4 |

Total amounts of adenosine nucleotide pools exhibited a decrease in the order of the none, the 8-azaguanine plus xanthine, the 8-azaguanine and the xanthine in the flavinogenic strain. This means that the pools showed a marked shrinkage upon the addition of 8-azaguanine, but the pools were recovered to a great extent by further addition of xanthine. However, with the addition of xanthine alone which caused high riboflavin production, the pools showed low values similar to those resulting from the addition of 8-azaguanine. Similar results in both cases is thought to be caused by the disappearance of the need for building blocks of riboflavin and energy source for flavinogenesis in the case of 8-azaguanine addition, and the exhaustion of these in the case of xanthine addition.

ATP pools are expressed as relative ratios against AMP pool as seen in Table 1. It is conceivable that with the addition of 8-azaguanine, the ATP synthesis decreased because there is little or no needs for an energy source for flavinogenesis when riboflavin formation is inhibited. However, upon further addition of xanthine, the ATP pool seems to be recovered simultaneously with the restoration of flavinogenesis by the effective utilization of exogenous xanthine. While with the addition of xanthine alone, the ATP pool showed marked low values. Accordingly, xanthine seems not to be effectively utilized for the synthesis of ATP, this being different from the case of the xanthine plus 8-azaguanine addition. The differences seem to result from the different needs for ATP as a regulator and an energy source in this mycelium, in the presence or absence of the inhibitor.

On the other hand, in the non-flavinogenic strain, a phenomenon contrary to that of the flavinogenic strain was observed in relation to the relative amounts
of adenosine nucleotide pools, although total adenosine nucleotide pools showed the same direction as that of flavinogenic strain.

5. Identification of the fractions accumulated in the presence of 8-azaguanine

Peaks I, II, III, IV, and V were respectively identified as uridine, 8-azaguanine, guanosine, 8-azaguanosine and 8-azaguanosine triphosphate by the methods described in Experimental.

Figure 7 shows the UV absorption spectra in 0.1 N hydrochloric acid of guanosine, 8-azaguanosine and 8-azaguanosine triphosphate, the spectra of the base moieties obtained by hydrolysis in 1 N hydrochloric acid and the spectra of the corresponding known purine bases.

These results indicate a good correspondences between the spectra of the known compounds and the spectra of the base moieties.

Furthermore, the Rf values in some solvents of 8-azaguanosine and 8-azaguanosine triphosphate on the paper chromatograms are demonstrated in Table 2. The relative molecular ratios of base, ribose and phosphate were 1.0 and 1.12 in 8-azaguanosine, and 1.0, 1.17 and 3.04 in 8-azaguanosine triphosphate.

DISCUSSION

Although it is well known that 8-azaguanine is a potent inhibitor of protein synthesis (24, 25), it is possible that in the case of non-growing cells (23), it functions as an analogue of guanine on the biosynthetic pathway of riboflavin. To elucidate this possibility, the effect of 8-azaguanine on riboflavin formation is examined in this paper.
BIOSYNTHESIS OF RIBOFLAVIN IN *E. ASHYI**I**

Table 2. *Rf* values of 8-azaguanosine and 8-azaguanosine triphosphate on paper chromatograms using three solvent systems.

Each compound was detected as the spots emitting blue-green fluorescence under an ultraviolet lamp. Solvent systems (ascending method) I: 1 M ammonium acetate buffer (pH 3.8)-99.5% ethanol (30:75), II: 0.1 M potassium phosphate buffer (pH 6.8)-ammonium sulphate-n-propanol (100 ml: 60 g: 2 ml), III: *n*-butanol-acetic acid-water (5:1:1).

| Solvent                | I  | II | III |
|-----------------------|----|----|-----|
| 8-Azaguanosine        | 0.64 | 0.18 | 0.20 |
| 8-Azaguanosine triphosphate | 0.13 | 0.24 | 0.05 |

The results obtained indicate that very low concentrations of 8-azaguanine result in a marked inhibition of riboflavin biosynthesis.

It has been known that this drug is a potent inhibitor of riboflavin formation by non-growing cells of *E. ashbyii*. However, the inhibition was completely overcome by supplementation of purines.

It can be assumed from these results that some step in the pathway from purine to riboflavin is completely blocked by 8-azaguanine or its derivatives.

On the other hand, it has been reported that 8-azaguanine inhibits the inter-conversions between purines and nucleotides (26–30) in addition to the inhibition of protein synthesis. For example, a feedback inhibition of the activity of phosphoribosyltransferase (26), non-competitive inhibition of xanthine oxidase (27) and pseudo-feedback inhibition of phosphoribosylpyrophosphate amidotransferase (28) have been found as a result of the action of 8-azaguanine. However, for the following reasons, it is not possible that the strong inhibition of riboflavin synthesis by 8-azaguanine is caused by blocking of the above reactions.

1. Purine is not the immediate precursor of flavinogenesis, instead, a type of nucleotide appears to be the more direct precursor (6). Furthermore, the purine derivatives in cells are also synthesized in the form of nucleotides. From these considerations, riboflavin production seems to be affected only a little even if xanthine oxidase and purine phosphoribosyltransferase is inhibited by 8-azaguanine.
2. The inhibition is completely recovered by the addition of various purines. Also from this result, the inhibition point does not seem to be in the relation of xanthine oxidase because the inhibition type of the oxidase by 8-azaguanine is of a non-competitive type and furthermore purine, which reverse the inhibition, is not only xanthine. (3) Phosphoribosylpyrophosphate amidotransferase may have no relations to the inhibition because of the deficiency of purine *de novo* synthesis in non-growing cells as examined with sulfanilamide (23).

Accordingly, it appears to be valid to consider that riboflavin production is specifically restricted because of the inhibition of the main pathway of riboflavin synthesis by this drug.
Next, fluctuations of nucleotide pools were examined in flavinogenic and non-flavinogenic strains in presence and absence of 8-azaguanine. The data obtained demonstrated that uridine, 8-azaguanine, guanosine, 8-azaguanosine and 8-azaguanosine triphosphate were noticeably accumulated in presence of 8-azaguanine. But uridine, 8-azaguanine and 8-azaguanosine desappeared and 8-azaguanosine triphosphate was markedly diminished by the addition of xanthine with 8-azaguanine, although large quantities of guanosine remained unchanged. From these facts, especially, the accumulation of a large amount of guanosine as a natural compound of this mycelium strongly supports the possibility that derivate of guanosine may be a direct precursor of riboflavin. Accordingly, the stimulatory effect of guanosine on flavinogenesis was examined in comparison with those of guanine and xanthine in non-growing cells.

However, even the addition of high concentrations of guanosine, up to 3 mM, caused the same stimulation of flavinogenesis as that of guanine and xanthine. Thus, it is evident that guanosine is not the immediate precursor of riboflavin as also verified in experiment with isotopically labelled guanosine by FORREST (31).

In the presence of 8-azaguanine, 8-azaguanosine triphosphate not 8-azaguanosine mono- and diphosphate accumulated in the flavinogenic strain. These results seem to support strongly the hypothesis that the most immediate precursor of riboflavin is guanosine triphosphate. If this suggestion is accepted, riboflavin biosynthesis should proceed through the cleavage reaction of the imidazole ring of guanosine triphosphate. Accordingly, these results can be schematically drawn as shown in Fig. 8.

![Fig. 8. Proposed pathway of riboflavin biosynthesis and inhibition mechanism of the pathway by 8-azaguanine. G: guanine, X: xanthine, 8-azaG: 8-azaguanine, GR: guanosine, GMP: guanosine monophosphate, GTP: guanosine triphosphate, TAP deriv.: 2,4,5-triaminopyrimidine derivative.](image-url)
However, as the deamination of guanosine triphosphate has to occur before its conversion to lumazine even if the synthetic mechanism of riboflavin is very similar to that of other pteridine, it is questionable whether the guanosine triphosphate cyclohydrolase (32, 33), detected in the biosynthetic pathway of the pteridine derivatives and folic acid, takes part in the above cleavage reaction or not.

Furthermore, the origin of the ribityl side chain and the ortho-xylene ring of riboflavin has also been obscure until now. Accordingly, it can be seen that many unknown problems are still involved in the riboflavin biosynthetic pathway.

REFERENCES

1) McNutt, W. S., J. Biol. Chem., 210, 511 (1954).
2) Brown, E. G., Goodwin, T. W., and Pendlington, S., Biochem. J., 61, 37 (1955).
3) Audley, B. G. and Goodwin, T. W., Biochem. J., 84, 587 (1962).
4) Mitsuda, H., Suzuki, Y., and Nakajima, K., Vitamins, 42, 380 (1970).
5) Mitsuda, H., Suzuki, Y., and Nakajima, K., Vitamins, 42, 386 (1970).
6) Mitsuda, H., and Nakajima, K., J. Vitaminol., 18, 137 (1972).
7) Dalal, H. and Gots, J. S., Biochim. Biophys. Res. Commun., 20, 509 (1965).
8) Mitsuda, H., Suzuki, Y., Tadera, K., and Kawai, F., J. Vitaminol., 12, 192 (1966).
9) Gu Moff, G. and Strenkoski, C. A., J. Biol. Chem., 241, 2220 (1966).
10) Shiota, T. and Palumbo, M. D., J. Biol. Chem., 240, 4449 (1965).
11) Levenberg, B. and Kaczmarek, D. K., Biochem. Biophys. Acta, 117, 272 (1966).
12) Burg, A. W. and Brown, G. W., Biochim. Biophys. Acta, 117, 275 (1966).
13) McNutt, W. S., J. Biol. Chem., 219, 365 (1956).
14) McNutt, W. S., J. Amer. Chem. Soc., 83, 2303 (1961).
15) Schlee, D., Reinbothe, H., and Fritsche, W., Z. Alg. Mikrobiol., 8, 127 (1968).
16) Mitsuda, H., Suzuki, Y., and Nakajima, K., J. Nutr. Sci. Vitaminol., 19, 29 (1973).
17) Dische, Z., J. Biol. Chem., 204, 983 (1953).
18) Ames, B. N. and Dubin, D. T., J. Biol. Chem., 235, 769 (1960).
19) Paladini, A. C. and Leloir, L. F., Biochem. J., 51, 426 (1952).
20) Bacon, J. S. P. and Edelmann, J., Biochem. J., 48, 114 (1951).
21) Bock, R. M., Morell, S. A., Ling, N. S., and Lipton, S. H., Arch. Biochem Biophys., 62, 253 (1951).
22) Davies, J. W. and Harris, G., Proc. Roy. Soc. (London), 537, 15113 (1960).
23) Mitsuda, H. and Nakajima, K., J. Vitaminol., 18, 131 (1972).
24) Roy-Burman, P., "Analogues of Nucleic Acid Components," Springer-Verlag Berlin, Heidelberg and New York, p. 28 (1970).
25) Kwan, S. W. and Webb, T. E., J. Biol. Chem., 242, 5542 (1967).
26) Berlin, R. D. and Stadtman, E. R., J. Biol. Chem., 241, 2679 (1966).
27) Feigelson, P. and Davidson, J., Cancer Res., 16, 352 (1956).
28) Ashton, D. M. and Wyngaarden, J. B., J. Biol. Chem., 239, 1560 (1964).
29) Levin, A. D. and Magasanik, B., Fed. Proc., 18, 272 (1959).
30) Feigelson, P. and Davidson, J. D., J. Biol. Chem., 223, 65 (1956).
31) Forrest, H. S. and McNutt, W. S., J. Amer. Chem. Soc., 80, 739 (1958).
32) Burg, A. W. and Brown, G. M., J. Biol. Chem., 243, 2344 (1968).
33) Cone, J. and Gu Moff, G., J. Biol. Chem., 246, 979 (1971).