THE IMMEDIATE NUCLEOTIDE PRECURSOR, GUANOSINE TRIPHOSPHATE, IN THE RIBOFLAVIN BIOSYNTHETIC PATHWAY

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Summary In the present paper, the nucleotide precursor of riboflavin was investigated by experiments with labeled purines using non-growing cells of *Eremothecium ashbyii*.

The added purines, at 10⁻⁴ M, were effectively incorporated into riboflavin at an early stage of riboflavin biosynthesis under the experimental conditions. In particular, both labeled xanthine and labeled guanine were specifically transported to guanosine nucleotides, GMP, GDP, GDP-Mannose and GTP, in the course of the riboflavin biosynthesis. A comparison of specific activities of labeled guanosine nucleotides and labeled riboflavin indicated that the nucleotide precursor of riboflavin is guanosine triphosphate. From the results obtained, a biosynthetic pathway of riboflavin is proposed under "DISCUSSION."

It has been established that purine is directly incorporated into riboflavin with the exception of C(8) of its imidazole ring (1-3). However, it remained unclear which purine is the most immediate precursor of riboflavin on the biosynthetic pathway, in spite of many laborious studies (1, 4-6). The present authors supposed that the different efficiencies of purines for flavinogenesis resulted from the different activities of purine uptake and interconversions of purines and nucleotides in various microorganisms and even in a same microorganism(7,8).

Accordingly, it is believed that added purines are introduced into the riboflavin biosynthetic pathway through different pathways. This supposes that not a purine precursor but a nucleotide precursor should be determined in the biosynthetic pathway of riboflavin. Previously, the authors presented evidence that guanosine triphosphate (GTP) is an immediate precursor of riboflavin in the form of nucleotide, from experiments with non-growing cells of *Eremothecium ashbyii*

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in the presence of a guanine analogue, 8-azaguanine (9, 10).

In the present paper, we furthermore tried to elucidate the possibility of GTP being a direct nucleotide precursor for flavinogenesis by experiments with labeled guanine and labeled xanthine in non-growing cells of *E. ashbyii*.

**MATERIALS AND METHODS**

*Microorganism.*  *Eremothecium ashbyii* IFO 0944 (flavinogenic strain) was obtained through the courtesy of the Institute for Fermentation, Osaka. The mycelia were maintained on an agar slant consisting of 1% glucose, 1% peptone, 0.1% yeast extract and 1.2% agar (pH 6.5) by periodically transplanting them to a fresh slant.

*Cultivation.* A fragment of the mycelia from a 7-day agar slant was transplanted into preculturual medium (50 ml) in a 300 ml-erlenmyer flask (containing the same nutrients as the slant except for agar), followed by cultivation for 1 day on a rotary shaker. Five milliliters of the precultural medium were transferred into the natural nutrient medium (500 ml, pH 5.5) in a 2 liter-erlenmyer flask containing 2% glucose, 1% peptone, 0.3% yeast extract, 0.2% KH₂PO₄, 0.1% NaCl, 0.01% MgSO₄·7H₂O and 1.8% Tween 80, then cultured for 1 day at 27°C in the dark on a rotary shaker (220 cycles/min). The mycelia (at the exponential phase of growth) were collected from the cultural fluid by filtration through a filter paper and then submitted to the experiments on non-growing cells of *E. ashbyii* (11).

*Non-growing cell incubation.* The mycelial pad obtained after enough washing with distilled water was suspended in 0.1 M phosphate buffer (pH 6.8) containing 0.5% glucose, and starved for 8 hr with moderate shaking. The suspension was filtered and adequately washed with distilled water. The washed mycelia (10 g wet weight) were suspended again in the same buffer (200 ml) containing 2.8×10⁻² M glucose and 10⁻⁴ M labeled purines (3.2×10⁵ cpm/µmole of [2-¹⁴C]guanine or 3.1×10⁶ cpm/µmole of [2-¹⁴C]xanthine which were obtained from CIS through JRIA). The purines were infiltrated into the mycelia under reduced pressure and the suspension was incubated for a definite time with mild shaking on a reciprocal shaker (12).

*Extraction of acid soluble nucleotides.* After the non-growing cell incubation, the suspension was filtered and the mycelia on a filter paper were washed well with distilled water. The mycelia were mixed in a mortar with 3 M HClO₄ (4 ml) and distilled water (20 ml), accompanied by hand grinding for several minutes to give an acid soluble nucleotides extract. The extract was neutralized with 3 M KOH and the precipitate (KClO₄) was removed by decantation. The clear supernatant obtained was used for separation of nucleotides.

*Separation and determination of nucleotides.* The acid soluble extracts (10 ml) were applied to a column (0.7×30 cm) of Dowex 1×2 (HCOO⁻, 200-
400 mesh) and separated into individual nucleotides by a gradient elution system in which the following solvents were successively run into a mixing chamber with a constant volume of distilled water (50 ml): first distilled water (150 ml) at a flow rate of 1.8 ml/5 min for washing, then (I) 0.5 M formic acid (100 ml), (II) 2 M formic acid (100 ml), (III) 2.5 M formic acid-0.18 M ammonium formate (100 ml), (IV) 3 M formic acid-0.31 M ammonium formate (150 ml) and (V) 3 M formic acid-2.2 M ammonium formate (100 ml), each at a flow rate of 0.9 ml/5 min (8). The eluate was collected in batches of 4 ml each and the absorbance of each fraction was determined at 260 nm, providing the elution pattern of nucleotides on the chromatogram. The nucleotides fractions were dried. Radioactivities of the dried materials were determined in a toluene scintillant containing 2 g of 2,5-diphenyloxazole (Nakarai Chemicals, Ltd.) and 0.05 g of 1,4-bis-2-(5-phenyloxazolyl)benzene (Packard Instrument Co., Inc.) per 500 ml solvent using a Packard Liquid Scintillation Counter (TRI-CARB 3320), showing the labeled pattern of nucleotides on the chromatogram. The amounts of guanosine nucleotides were calculated from the volume and the absorbance of each nucleotide fraction using 11.8 as their millimolar extinction coefficient at 260 nm.

**Determination of riboflavin.** For determination of riboflavin content and radioactivity, the following procedures were undertaken. The cell suspension (10 ml) after the non-growing cell incubation was heated in a water bath at 80°C for 10 min and centrifuged. The obtained supernatant was applied to a column (1×10 cm) first packed with Dowex 50W (H+, 200-400 mesh) to a height of 8 cm and above it with Dowex 1×2 (Cl−, 200-400 mesh) to a further 2 cm height, followed by elution with distilled water at a flow rate of 3 ml/5 min. The fractionated riboflavin solution underwent the determinations of absorbance at 450 nm and radioactivity by the same methods as described above. The riboflavin contents were calculated from the absorbance at 450 nm using 11.2 as the millimolar extinction coefficient.

**RESULTS**

1. **Acid soluble nucleotides after 12 hr incubation with non-growing cells in the presence of [2-14C]guanine or [2-14C]xanthine**

Figure 1 shows that large amounts of NAD, GDP-M, UDP-Gal and ATP were observed but the clear separation of UMP and ADP, UDP-Gal and GDP or GTP and probably UTP was not attained on these chromatograms. However, the elution patterns and the labeled patterns of nucleotides interestingly showed quite a similarity with the addition of [2-14C]guanine or [2-14C]xanthine.

In addition, labeled guanine or xanthine were specifically incorporated into guanosine nucleotides, GpA³ (13, 14), GMP, GDP-M and GTP in the 12 hr incubation with non-growing cells of E. ashbyii, during which riboflavin forma-
Fig. 1. Column chromatography on Dowex 1×2 (HCOO⁻) of acid soluble nucleotides from non-growing cells of Eremothecium ashbyii in the 12 hr incubation. Purines were added at $10^{-3}$ M (6.42×$10^6$ cpmp of guanine or 6.25×$10^6$ cpmp of xanthine) to the non-growing cell medium and incubated for 12 hr on a reciprocal shaker. The mycelia were filtered, washed and extracted with perchloric acid, giving the acid soluble nucleotides. The extracts were applied to a column of Dowex 1×2 (HCOO⁻) and developed by the indicated solvent systems: I) 0.5 M formic acid (100 ml), II) 2 M formic acid (100 ml), III) 2.5 M formic acid + 0.18 M ammonium formate (100 ml), IV) 3 M formic acid + 0.31 M ammonium formate (150 ml) and V) 3 M formic acid + 2.2 M ammonium formate (100 ml) in this order.

The accumulation proceeded exponentially. Furthermore, highly effective incorporation of labeled purines into GDP-M and GTP of the guanosine nucleotides was observed in both cases.

The amounts and radioactivities of these nucleotides were determined and the results are given in Table 1. Specific activities increased in the order of GMP,
Table 1. Incorporation of [2-14C]guanine and [2-14C]xanthine into guanosine nucleotides and riboflavin in the 12 hr incubation with non-growing cells of *E. ashbyii*. The values in the table were calculated from the results of Fig. 1. Although GTP could not be clearly separated on the chromatograms as seen in Fig. 1, the indicated values were ascertained by another chromatography on Dowex 1×2 (Cl⁻) which was developed by the use of hydrochloric acid as solvent. The specific activities of initially added purines were $3.2 \times 10^5$ cpm/μmole for guanine and $3.1 \times 10^5$ cpm/μmole for xanthine.

| Guanine     | GMP  | GDP  | GDP-M | GTP  | $B_2$ |
|-------------|------|------|-------|------|-------|
| S.A. (cpm/μmole) | $8.9 \times 10^4$ | $13 \times 10^4$ | $11 \times 10^4$ | $18 \times 10^4$ |
| Total activity (cpm) | $1.4 \times 10^4$ | $2.4 \times 10^4$ | $51 \times 10^4$ | $18 \times 10^4$ |
| Total amount (μmole) | 0.16 | —    | 3.96  | 1.54 | 6.48  |

| Xanthine    | GMP  | GDP  | GDP-M | GTP  | $B_2$ |
|-------------|------|------|-------|------|-------|
| S.A. (cpm/μmole) | $7.9 \times 10^4$ | $13 \times 10^4$ | $10 \times 10^4$ | $20 \times 10^4$ |
| Total activity (cpm) | $1.3 \times 10^4$ | $1.8 \times 10^4$ | $45 \times 10^4$ | $13 \times 10^4$ |
| Total amount (μmole) | 0.16 | —    | 3.39  | 1.32 | 6.67  |

GTP, GDP-M and $B_2$ in the presence of [2-14C]guanine or [2-14C]xanthine. Furthermore, the specific activity of GpA was lower than that of GMP. The specific activities of GMP and GTP in the guanine addition were higher than those in the xanthine addition, whereas the specific activity of riboflavin in the guanine addition was lower than that in the xanthine addition. These results indicate that xanthine is more effectively incorporated into riboflavin than guanine. Furthermore, a comparison of the specific activities and the total activities of the initially added purines with those of the riboflavin produced indicates that the incorporation of added purines into riboflavin was diluted to about two-thirds, while about 20% of the added purines were already incorporated into riboflavin during the 12 hr incubation in both additions.

2. Incorporation of [2-14C]guanine into guanosine nucleotides during the non-growing cell incubation

The elution and labeled patterns of nucleotides were examined at 6 hr, 12 hr and 24 hr after the addition of [2-14C]guanine during the non-growing cell incubation. The results are given in Fig. 2, which indicates that the general profiles are quite similar to those of Fig. 1. The amounts and radioactivities of nucleotides gradually decreased with the increased incubation time except for those of GpA. On these chromatograms, the higher incorporation of labeled guanine into GDP-M and GTP than GMP and GDP were observed during the incubation.

Total activities of these radioactive guanosine nucleotides are depicted respectively in Fig. 3. The total activity of riboflavin shows that added purines were quite rapidly incorporated into riboflavin in the incubation period of 0 hr to 6 hr, but thereafter proceeded gradually until 24 hr. On the other hand, total radioactivities of GDP-M, GTP, GDP and GMP indicate that labeled
Fig. 2. Column chromatography on Dowex 1×2 (HCOO⁻) of acid-soluble nucleotides from non-growing cells of E. ashbyii in the 6 hr, 12 hr and 24 hr incubations. Guanine was added at 10⁻⁴ M (6.42×10⁶ cpm) to the non-growing cell medium and incubated for the indicated times. The column systems and other experimental conditions were the same as those in Fig. 1. The elution patterns are shown by open circles and the labeled patterns by closed circles in the figure.
guanine was promptly incorporated into these nucleotides during the 0–6 hr incubation in the same way as into riboflavin, but their values decreased markedly with further incubation until 24 hr, showing the inverse behavior of that of ribo-

Fig. 3. Behavior of total radioactivities of guanosine nucleotides and riboflavin. The values of nucleotides were calculated from the results in Fig. 2 and those of riboflavin were determined from the results obtained by chromatography on a double-layered column of Dowex 1 × 2 (Cl⁻) and Dowex 50W × 4 (H⁺), the details of which have been described under MATERIALS AND METHODS.

Fig. 4. Behavior of specific activities of guanosine nucleotides and riboflavin. The values for guanosine nucleotides and riboflavin were calculated from the results in Fig. 2 and Fig. 3. The specific activity of riboflavin is expressed by horizontal straight lines as the mean values, which were obtained by dividing the total activity of riboflavin produced in a 6–12 hr incubation or a 12–24 hr incubation by the amounts of riboflavin for the corresponding incubation periods.
flavin. In these experiments, the values of GDP-M and GTP were much higher than those of GDP and GMP during the incubation.

The specific activities of guanosine nucleotides and riboflavin were calculated from the results of Fig. 2 and Fig. 3 and depicted in Fig. 4. Riboflavin contents increased linearly with increased incubation time, during which the specific activities of GDP-M, GTP, GMP and riboflavin decreased with increased incubation time, probably providing maximum values of their specific activities in the incubation period between 0 hr and 6 hr. The specific activity of GDP-M was higher and that of GMP was lower than that of riboflavin, while the specific activity of GTP was almost similar to that of riboflavin throughout the incubation. These results indicate that the direct nucleotide precursor is guanosine triphosphate but not guanosine diphosphate mannose in the biosynthetic pathway of riboflavin.

**DISCUSSION**

There are many reports that purines are directly incorporated into the riboflavin skeleton with the exception of C(8) of the imidazole ring in the riboflavin biosynthesis (1-3). This fact was verified also by experiments with labeled glycine (15) and labeled formate (16), being building blocks for the de novo synthesis of purine base. Furthermore, the question of which purine is the most efficient precursor of riboflavin has been examined in various microorganisms (1, 4-6). But the results obtained were not necessarily consistent. Previously, the present authors tried to determine the purine precursor using non-growing cells of a high flavinogenic mold, *Eremothecium ashbyii*. The results demonstrated that the different efficiencies of purines for flavinogenesis would depend upon the discrepancies between salvage pathways of exogenous purines and interconversion pathways of purines or purine nucleotides in different microorganisms or sometimes even in a same microorganism (7). However, the strong possibility was found that of the purines xanthine is the most immediate precursor of riboflavin, and is transferred to riboflavin via guanosine monophosphate in the experiments with growing or non-growing cells of *E. ashbyii* (7, 8).

Recently, BACHER and MAILÁNDER (17) showed that not [2-14C]xanthine but [2-14C]guanine was incorporated into riboflavin in a mutant, *Aerobacter aerogenes*, lacking XMP aminase, in which the conversion of xanthine to guanosine monophosphate is impossible. BAUGH and KRUMDIECK (18) further indicated that [2-14C]guanine was converted to riboflavin without dilution whereas [2-14C]xanthine was not converted to riboflavin, using an antibiotic decoyinine to inhibit the formation of GMP from XMP in Corynebacterium sp. which lacks GMP reductase.

These results are compatible with our previous data (7, 8). Furthermore, the authors found the possibility that guanosine triphosphate is an immediate precursor of riboflavin in the nucleotide form, using a guanine analogue 8-aza-guanine in non-growing cells of *E. ashbyii* (9, 10). This is supported by the fact that guanosine triphosphate is a precursor of folic acid (19–21) and toxoflavin (22),
which are biosynthesized through a pteridine having the same or almost similar ring as 6,7-dimethyl-8-ribityllumazine, a precursor of riboflavin.

In the present experiments, labeled guanine and xanthine were efficiently transferred to riboflavin under the experimental conditions with non-growing cells of *E. ashbyii*, as shown in Table 2. The incorporation ratios of exogenous purines into riboflavin showed high values of up to 81.8% of the produced riboflavin in the 6 hr incubation, but thereafter the incorporation ratios gradually decreased, being 37.3% in the 24 hr incubation, in spite of the fact that incorporation continued to increase with increasing incubation time. The decrease of incorporation ratios at the later stage of flavinogenesis is considered to have resulted from sufficient supplementation with an endogenous substrate of riboflavin. The endogenous substrate sources of riboflavin have not been studied. As to the problem, the results obtained suggest that the endogenous sources are DNA and RNA, especially RNA in such a high flavinogenic mold as *Eremothecium ashbyii*, because the dilution of the incorporation ratio begins simultaneously with the initiation of the lysis of the mold in the present experiment and also in the experiment with growing cells (11).

On the other hand, the labeled purines, xanthine and guanine, were specifically incorporated into guanosine nucleotides, especially GDP-M and GTP in non-growing cells of the mold. This indicates that the non-growing cells of *E. ashbyii* behave like a mutant lacking GMP reductase which converts GMP to IMP, although the growing cells of the mold revealed the activity of GMP reductase (23).

The characteristic attitude of the mold strongly suggests that riboflavin biosynthesis is closely coupled with guanosine nucleotide pathways consisting of guanine uptake and phosphorylation of GMP to GTP. Furthermore, a comparison of specific activities of guanosine nucleotides, GMP, GTP and GDP-M,
Fig. 5. Proposed pathway of riboflavin biosynthesis. The open lines exhibit the inhibition of the biosynthetic pathway and the closed lines show the directions of the pathway. 8-Ribityllumazine and 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-8-ribityllumazine are the forms of the intermediate (25) or the by-product (26), 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine isolated from the mycelia of *Eremothecium ashbyii* in the presence of glyoxal or dimeric diacetyl as trapping agents.
and those of riboflavin shows that guanosine triphosphate is a direct nucleotide precursor of riboflavin.

Accordingly, although GTP is a common precursor of riboflavin, folic acid (19–21) and other pteridines (22, 24), the deamination of the amino group at C–2 of the purine ring of GTP and the reduction of the ribose moiety of GTP to ribitol are involved in the formation of riboflavin. In these points, the biosynthetic pathway of riboflavin differs from those of folic acid and other pteridines. The authors are trying to elucidate these problems in the biosynthetic pathway of riboflavin.

The proposed biosynthetic pathway of riboflavin is depicted in Fig. 5. In the figure it is concluded that after the rupture of the imidazole ring, GTP is converted to riboflavin through an immediate intermediate, 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (25), which is also the by-product of the riboflavin synthetase reaction (26) and is probably reutilized for the formation of riboflavin in vivo (27). The pyrimidine which is the precursor or the by-product on the pathway was isolated in the form of 8-ribityllumazine or 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-8-ribityllumazine from the mold or the enzyme reaction mixture, using glyoxal or dimeric diacetyl as trapping agents in these experiments.

REFERENCES

1) McNutt, W. S. (1954): The direct contribution of adenine to the biogenesis of riboflavin by Eremothecium ashbyii. J. Biol. Chem., 210, 511–519.
2) McNutt, W. S. (1956): The incorporation of the pyrimidine ring of adenine into the isoaloxazine ring of riboflavin. J. Biol. Chem., 219, 365–373.
3) McNutt, W. S. (1961): The incorporation of the four nitrogen atoms of purines into the pyrimidine and pyrazine rings of riboflavin. J. Amer. Chem. Soc., 83, 2303–2307.
4) McLaren, J. A. (1952): The effects of certain purines and pyrimidines upon the production of riboflavin by Eremothecium ashbyii. J. Bacteriol., 63, 233–241.
5) Brown, E. G., Goodwin, T. W., and Jones, O. T. G. (1958): Studies on the biosynthesis of riboflavin. 4. Purine metabolism and riboflavin synthesis in Eremothecium ashbyii. Biochem. J., 68, 40–49.
6) Osman, H. G., and Soliman, M. H. M. (1960): Biosynthesis of riboflavin by Eremothecium ashbyii. II. Effect of some amino acids, ureido acids, hydantoins and some other nitrogenous compounds on the growth and vitamin B2 production. Biochem. Zeit., 333, 351–360.
7) Mitsuda, H., and Nakajima, K. (1972): Stimulatory effects of purines on flavinogenesis by non-growing cell of Eremothecium ashbyii. J. Vitaminol., 18, 137–147.
8) Mitsuda, H., Suzuki, Y., and Nakajima, K. (1973): Fluctuation of the nucleotide pools of flavinogenic and non-flavinogenic strains of Eremothecium ashbyii. J. Nutr. Sci. Vitaminol., 19, 29–42.
9) Mitsuda, H., and Nakajima, K. (1973): Effects of 8-azaguanine on riboflavin production and on nucleotide pools in non-growing cells of Eremothecium ashbyii. J Nutr. Sci. Vitaminol., 19, 215–227.
10) Mitsuda, H., and Nakajima, K. (1975): Guanosine nucleotide precursor for flavinogenesis of Eremothecium ashbyii. J. Nutr. Sci. Vitaminol., 21, 331–345.
11) Mitsuda, H., Suzuki, Y., and Nakajima, K. (1970): Effects of Non-ionic surface active agents on riboflavin production by Eremothecium ashbyii. Vitamins (in Japanese), 42,
12) MITSUDA, H., and NAKAJIMA, K. (1972): Development of specific experimental systems for flavinogenesis using non-growing cell of *Eremothecium ashbyii*. *J. Vitaminol.*, 18, 131-136.

13) MITSUDA, H., NISHIKAWA, Y., and NAKAJIMA, K. (1974): A new compound accumulated in non-growing cells of *Eremothecium ashbyii* by the addition of caffeine. *Vitamins* (in Japanese), 48, 111-120.

14) MITSUDA, H., NISHIKAWA, Y., and NAKAJIMA, K. (1976): Formation of guanine ribonucleotidyl-(3'-5')-adenosine in a flavinogenic strain of *Eremothecium ashbyii*. *J. Nutr. Sci. Vitaminol.*, 22, 115-133.

15) HOWELLS, D. J., and PLAUT, G. W. E. (1965): Biosynthesis of riboflavin by a purine-requiring mutant strain of *Escherichia coli*. *Biochem. J.*, 94, 755-759.

16) KLUNGSØYR, L. (1954): The biosynthesis of riboflavin in *Eremothecium ashbyii*. *Acta Chem. Scand.*, 8, 723-727.

17) BACHER, A., and MAILÅNDER, B. (1973): Biosynthesis of riboflavin. The structure of the purine precursor. *J. Biol. Chem.*, 248, 6227-6231.

18) BAUGH, C. M., and KRUMDIEK, CL. (1969): Biosynthesis of riboflavin in *Corynebacterium Species*: the purine precursor. *J. Bacteriol.*, 98, 1114-1119.

19) BURG, A. W., and BROWN, G. W. E. (1968): The biosynthesis of folic acid. VIII. Purification and properties of the enzyme that catalyzes the production of formate from carbon atom 8 of guanosine triphosphate. *J. Biol. Chem.*, 243, 2349-2358.

20) CONE, J., and GURROFF, G. (1971): Partial purification and properties of guanosine triphosphate cyclohydrolase, the first enzyme in pteridine biosynthesis from *Comamonas sp.* (ATCC 11299a). *J. Biol. Chem.*, 246, 979-985.

21) MITSUDA, H., SUZUKI, Y., TADERA, K., and KAWAI, F. (1966): Biochemical studies on pteridines in plants. II. Biogenesis of folic acid in green leaves: enzymatic synthesis of dihydropterolic acid from guanosine compounds and mechanism of its synthetic pathway. *J. Vitaminol.*, 12, 192-204.

22) LEBENBERG, B., and KACZMAREK, D. K. (1966): Enzymatic release of carbon atom 8 from guanosine triphosphate, an early reaction in the conversion of purines to pterines. *Biochem. Biophys. Acta*, 117, 272-275.

23) AUDLEY, B. G., and GOODWIN, T. W. (1962): Studies on the biosynthesis of riboflavin. 7. The incorporation of adenine and guanine into riboflavin and into nucleic acid purines in *Eremothecium ashbyii* and *Candida flarea*. *Biochem. J.*, 84, 587-592.

24) WEYGAND, F., SIMON, H., DAHMS, G., WALDSCHMIDT, M., SCHLIEP, H. J., and WACKER, H. (1961): Über die biogenese des leucopterins. *Angew. Chem.*, 73, 402-407.

25) MITSUDA, H., and NAKAJIMA, K. (1976): Isolation of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine from a high flavinogenic mold *Eremothecium ashbyii*. *J. Nutr. Sci. Vitaminol.*, 22, 307-312.

26) MITSUDA, H., NADAMOTO, T., and NAKAJIMA, K. (1976): Identification of the second product of the riboflavin synthetase reaction. *J. Nutr. Sci. Vitaminol.*, 22, 381-387.

27) MITSUDA, H., NADAMOTO, T., and NAKAJIMA, K. (1976): Reutilization of by-product for riboflavin formation in the riboflavin synthetase reaction. *J. Nutr. Sci. Vitaminol.*, 22, 67-70.