NUCLEOTIDE PRECURSOR IN RIBOFLAVIN
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Although it is well known through many reports (1-4) that purines are precursors of riboflavin it is still obscure as to which purine is an immediate precursor. Previously, we reported that the efficiency of purines for flavinogenesis may depend on the activities of purine uptake and the interconversion of purines and nucleotides in different microorganisms and also in the same microorganism (5). However, we discovered the possibility that various purines, especially xanthine, added exogenously are incorporated into riboflavin through guanosine triphosphate by experiments with a guanine analogue, 8-azaguanine, in non-growing cells of *Eremothecium ashbyii* (6, 7). BAUGH (8) and BACHER (9, 10) suggested that an unidentified guanine derivative is an immediate precursor of riboflavin by radioisotope experiments using mutants, *Aerobacter aerogenes* and *Corynebacterium* sp. The results are compatible with our results described above. In the present paper, we further investigated the possibility of guanosine triphosphate being a direct nucleotide precursor of riboflavin by experiments with labelled guanine in non-growing cells of *E. ashbyii*.

Eremothecium ashbyii cells which were grown on a natural nutrient medium for 24 hr were submitted to the non-growing cell experiments reported previously (11). After non-growing cell incubation with labelled guanine for 12 hr, the mycelia obtained were extracted with perchloric acid solution and the acid soluble fluid was subjected to column chromatographic analysis on Dowex 1×2 (HCOO⁻) as described in a previous report (7). Furthermore, the incorporation of labelled guanine into GTP was confirmed also by Dowex 1×2 (Cl⁻) column chromatography, which was developed by a linear gradient elution with 0.1 N HCl solution. Radioactivity of isolated nucleotides was counted with a Packard Liquid Scintillation Counter (TRI-CARB 3320).

Determination of riboflavin content and radioactivity was conducted as follows. The cell suspension at the end of the incubation period (12 hr) was

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Abbreviations: G_pA; guanine ribonucleotidyl-(3'-5')-adenosine; GDP-M; guanosine diphosphate mannose; UDP-Gal, uridine diphosphate galactose.

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Fig. 1. Dowex 1 $\times$ 2 (HCOO⁻) column chromatography of acid soluble nucleotides from non-growing cells of *Eremothecium ashbyii* at the 12th hr of incubation.

Guanine was added at 10^{-4} M (6.42×10^6 cpm) 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 to give acid soluble nucleotides. The extracts were applied to a Dowex 1 $\times$ 2 (HCOO⁻) column 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.

heated in a water bath at 80°C for 10 min and centrifuged. The clear fluid was applied to a column (1 $\times$ 10 cm) first packed with Dowex 50W $\times$ 4 (H⁺, 200–400 mesh) to a height of 8 cm and then with Dowex 1 $\times$ 2 (Cl⁻, 200–400 mesh) to a height of 2 cm, and was then eluted with distilled water. The absorbance of the riboflavin fraction was determined at 450 nm and radioactivity was determined by the same method described above.

The open circles in Fig. 1 show the nucleotide elution pattern of the mycelia incubated for 12 hr with labelled guanine. Clear separation of NAD and AMP, UMP and ADP, UDP-Gal and GDP or GTP and probably UTP could not be achieved on the chromatogram. However, the fractions of NAD, AMP, G_pA (12), GMP, ADP, GDP-M, UDP-Gal, ATP and GTP were clearly observed in various amounts. On the other hand, it was of great interest to see, [2-¹⁴C]guanine incorporated into only guanosine nucleotides, such as G_pA, GMP, GDP-M, GDP and GTP on the active flavinogenesis, as shown by closed circles in Fig. 1. This indicates that the non-growing cells of *E. ashbyii* are like a mutant lacking GMP reductase (EC 1.6.6.8) which converts GMP to IMP in contrast to the growing cells of the mold (13). Furthermore, it should be noted that the total radioac-

Table 1. Incorporation of [2-¹⁴C]guanine into nucleotides and riboflavin in *Eremothecium ashbyii*. The values were calculated from the results of Fig. 1 and the results from another Dowex 1×2 (Cl⁻) chromatography.

	[2- ¹⁴ C]Guanine ^a	GMP	GDP	GTP	GDP-M	B ₂
Amounts (μmoles)	20.0	0.08	0.22	1.34	3.11	7.55
Total activity (cpm × 10 ⁻³)	6,420	7.5	21.9	145.9	407	1,470
Specific activity (cpm/μmoles × 10 ⁻³)	321	96	99	110	131	195

^a Values of initially added guanine.

tivity of GTP shows the highest value of guanosine nucleotides (GMP, GDP and GTP). These results were also ascertained by Dowex 1×2 (Cl⁻) column chromatography which indicated more clearly the incorporation of [2-¹⁴C]guanine into GTP.

The amounts, total activity and specific radioactivity of GMP, GDP, GTP and GDP-M, being radioactive nucleotides on the chromatogram, were calculated from the results of Fig. 1 and the results from the Dowex 1×2 (Cl⁻) chromatography. The results are given in Table 1, and indicate that the amounts of GTP were highest among the guanosine nucleotides, with the exception of GDP-M, and their radioactivity increased with the amounts of each guanosine nucleotide. The values of specific radioactivity increased successively in the order of GMP, GDP, GTP, GDP-M and riboflavin. The results indicate that a large amount of added guanine was already incorporated into riboflavin at the 12 hr incubation when riboflavin biosynthesis reached the exponential stage. This was easily determined also by comparison of the specific activity of the initially added guanine with that of riboflavin produced. Under experimental conditions, the specific activity of GTP exhibited the highest value of guanosine nucleotides with the exception of GDP-M.

In the present experiments with non-growing cells of *Eremothecium ashbyii*, [2-¹⁴C]guanine was incorporated only into guanosine nucleotides at the exponential stage of flavinogenesis, showing the highest incorporation into GTP of guanosine nucleotides except for GDP-M. It is well known that GDP-M is directly formed from GTP and mannose-1-phosphate in cells (14). Thus, it is quite conceivable that the high radioactivity of GDP-M was obtained under physiological conditions in which [2-¹⁴C]guanine was actively incorporated into riboflavin through guanosine triphosphate. Accordingly, it is concluded that guanosine triphosphate is an immediate nucleotide precursor of riboflavin.

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