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BIOSYNTHESIS OF RIBOFLAVIN: ENZYMATIC CONVERSION OF 5-AMINO-2,4-DIOXY-6-RIBITYLAMINOPYRIMIDINE TO 6,7-DIMETHYL-8-RIBITYLLUMAZINE

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SUMMARY: The conversion of 5-amino-2,4-dioxy-6-ribitylaminopyrimidine (ADRAP) to 6,7-dimethyl-8-ribityllumazine, the immediate precursor of riboflavin, can take place in the presence of an extract of Escherichia coli. The extract can be separated into 2 protein fractions, both of which are needed for the transformation, and pyridine nucleotide, supplied most efficiently as NAD^+, is required. Since no carbon source other than ADRAP is needed, we conclude that 2 moles of ADRAP are used in the transformation, one to serve as donor of the 4 extra carbons needed for the transformation, and one to serve as the acceptor.

INTRODUCTION: The biosynthesis of riboflavin is thought to proceed via the individual enzymatic reactions shown in Fig. 1. Formulation of this pathway is based on: (a) an analysis by Bacher and Lingens (1) of the excretion products of a series of riboflavin-requiring mutants of Saccharomyces cerevisiae, (b) enzyme work from this laboratory (2,3), and (c) the existence of riboflavin synthetase (see Plaut (4)), which catalyzes the last enzymatic step. The first enzyme of the pathway, named GTP cyclohydrolase II, was discovered and purified from Escherichia coli by Foor and Brown (2); the next two enzymes, which catalyze reactions 2 and 3 (deamination and reduction, respectively), were purified from E. coli by Burrows and Brown (3). There is some evidence that in yeast (1) and Ashbya gossypii (5) reactions 2 and 3 are reversed in the pathway (i.e., that reduction precedes deamination). The portion of the pathway which until recently has remained unclear is the conversion of Compound IV (Fig. 1) to 6,7-dimethyl-8-ribityllumazine (DMRL, Compound V). The source and nature of the 4 extra carbons needed for this transformation has remained in doubt.

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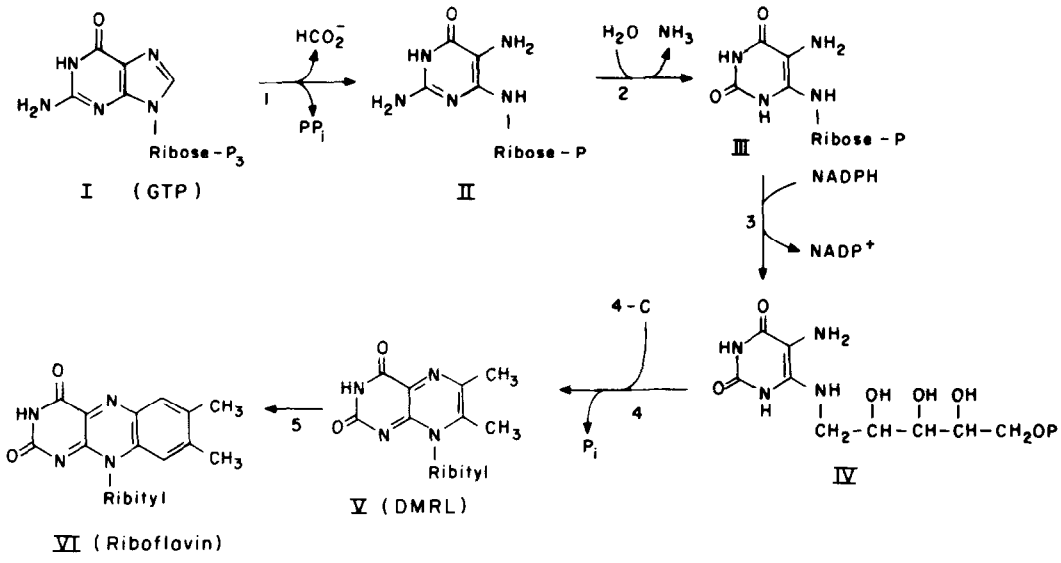

Fig. 1. Enzymatic reactions thought to be involved in the biosynthesis of riboflavin.

The present work reports on the enzymatic conversion of Compound IV to DMRL and provides some information on the probable source of these carbons.

MATERIALS AND METHODS: 5-Nitroso-2,4-dioxy-6-ribitylaminopyrimidine was synthesized as described by Maley and Plaut (6). This compound was reduced to 5-amino-2,4-dioxy-6-ribitylaminopyrimidine (ADRAP) by catalytic hydrogenation (platinum oxide catalyst) immediately before ADRAP was to be used as substrate.

Purified deaminase and reductase (the enzymes that catalyze reactions 2 and 3, respectively, in Fig. 1) were prepared from E. coli as described by Burrows and Brown (3). GTP cyclohydrolase II was prepared as described by Foor and Brown (2). Frozen E. coli cells were obtained from Grain Processing Corp. Extracts were prepared and other materials were obtained as described earlier (3).

Products of enzymatic action (riboflavin and DMRL) were separated and determined quantitatively by high pressure liquid chromatography (HPLC). The system, supplied by Waters Associates, consisted of two model 6000A pumps, a U6K injector, a model 660 solvent programmer, and a Bondapak C-18 column protected by a guard column filled with Bondapak C10/Corasil. Detection was by a model 420 fluorescence detector, and a Houston Instruments Omni Scribe dual pen recorder. Excitation was at 360 nm and fluorescence was measured at 460 nm and higher. All solvents were HPLC grade from Fisher Scientific. Water was purified with the use of a Millipore 4 bowl MilliQ purification system.

Compound IV, labeled with ^{14}C, was generated by incubation of [U-^{14}C] GTP with purified preparations of GTP cyclohydrolase II, deaminase, and reductase. For this purpose, a reaction mixture was prepared to contain 125 mM Tris-HCl buffer (pH 8.0), 2.5 mM $MgCl_2$, 25 mM mercaptoethanol, 25 µM [U-^{14}C]GTP (1.2×10^6 cpm) and 4 milliunits of GTP cyclohydrolase in a total volume of 0.18 ml. This mixture was incubated for 15 minutes at 39° after which purified deaminase(1 µg of protein)and EDTA (final concentration, 2.4 mM) were added and the mixture was reincubated for 10 minutes at 39°.

Finally, NADPH (final concentration, 1 mM) and purified reductase (1 µg of protein) were added and the mixture was incubated for an additional 10 minutes at 39°. After the final incubation, $MgCl_2$ was added to give a final concentration of 15 mM. This solution, which contained approximately 5 µM Compound IV (generated from GTP), was used as a source of Compound IV in some of the experiments to be described.

RESULTS AND DISCUSSION: A portion (0.025 ml) of the solution containing radioactive Compound IV was incubated with E. coli crude extract (7 mg of protein) for 30 minutes at 37° and after the addition of standard DMRL and riboflavin the incubated material was heated at 70° for 5 min, centrifuged, and the resulting supernatant solution analyzed for the production of radioactive DMRL and riboflavin by paper chromatography and thin-layer chromatography. For this purpose, the material was applied to a strip (3 x 46 cm) of Whatman 3 MM paper and the chromatogram was developed (descending fashion) with 60% t-butyl alcohol. The resulting fluorescent bands corresponding to standard DMRL and riboflavin were excised and the material from each band was eluted with water and subjected to two-dimensional thin-layer chromatography on cellulose plates with 20% ethanol as the solvent in the first dimension and 3% NH_4Cl in the second. The developed plates were analyzed for radioactivity by radioautography. The results showed that the fluorescent areas corresponding to DMRL and riboflavin were radioactive. Since no obvious source of the 4 carbons necessary to convert Compound IV to DMRL had been added to the reaction mixture, the supposition is that the extra carbons were supplied by the components of the reaction mixture; i.e., the Compound IV preparation, which would be expected to be contaminated with unreacted GTP and metabolites of GTP, or small molecules that might be present in the crude extract. To investigate these possibilities further, the crude extract was brought to 80% saturation with ammonium sulfate and the resulting protein fraction was dissolved in buffer (pH 8.0) containing 40 mM Tris-HCl, 5 mM $MgCl_2$ and 10% glycerol. After dialysis against the same buffer the solution was subjected to filtration at 4° through a column (2.5 x 110 cm) of Ultrogel ACA 44 (obtained from LKB Instruments). Analyses of the column fractions

indicated that two protein fractions (Fractions A and B) were both needed for the conversion of Compound IV to DMRL. Since Fraction B was contaminated with riboflavin synthetase, a mixture of DMRL and riboflavin was detected as products. Again, no source of the extra 4 carbons needed to be supplied except for the Compound IV preparation. The possibility that the extra carbons could be supplied as contaminants in the enzyme preparation seemed less likely because of the treatment given the preparation.

We also found that treatment of the Compound IV preparation with alkaline phosphatase (E. coli, Sigma Type III-S), prior to the incubation to produce DMRL, did not diminish the amount of DMRL and riboflavin produced. This suggested that dephosphorylation of Compound IV probably occurs prior to the synthesis of DMRL. The dephosphorylated product is 5-amino-2,4-dioxy-6-ribitylaminopyrimidine (ADRAP). This compound was synthesized (see Materials and Methods) and tested for its ability to be converted enzymatically to DMRL and riboflavin. For this purpose, a reaction mixture was prepared to contain (in total volume of 0.135 ml) 80 μM ADRAP, 50 μM NADPH, 50 μM NAD, 4 mM $MgCl_2$, 8.4% glycerol, 34 mM Tris-HCl (pH 8.0) and Fractions A and B (ca. 1.5 mg protein of each). Incubation was for 1 hour at 37°. The incubated reaction mixture was subjected to analysis by HPLC in a 3% to 50% methanol gradient with a flow rate of 1.5 ml per minute. Two fluorescent products were separated (Fig. 2) whose elution patterns corresponded exactly to those of standard DMRL and riboflavin. The fractions containing the putative riboflavin were combined and the material was subjected to 2-dimensional thin-layer chromatography as described previously. The results indicated that the fluorescent material migrated as standard riboflavin. The maximum yield of riboflavin plus DMRL from ADRAP that we have observed is 30%.

Additional experiments have shown that a source of pyridine nucleotide is needed for the production of riboflavin from ADRAP (Table 1). This requirement can be met with relatively high concentrations of NADPH, $NADP^+$, NADPH and NAD^+, but NAD^+ is used most effectively at lower concentrations

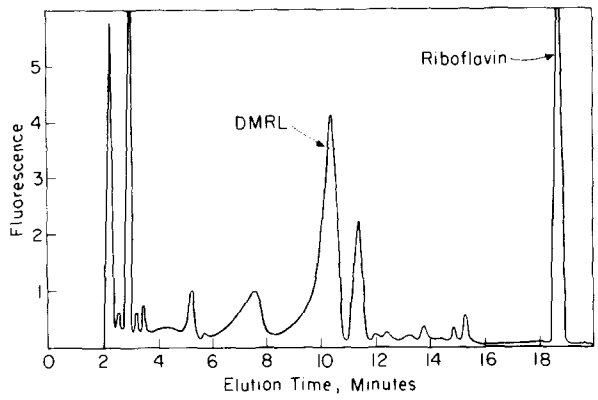

Fig. 2. Separation of fluorescent components of an incubated reaction mixture by HPLC. Fluorescence is presented in arbitrary units. The unlabeled fluorescent peaks have not been identified with elution patterns of any known compounds.

(Table 1). The data indicate that NAD^+ acts catalytically and, therefore, suggest that any NADH that may be generated by an oxidation is consumed in a subsequent reduction in the conversion of ADRAP to DMRL.

These observations indicate that the source of the extra 4 carbons needed to convert ADRAP to DMRL is ADRAP. The likely possibility is that the extra carbons are derived from the 5-carbon ribityl group of ADRAP, a suggestion made earlier by Bresler et al. (7) based on their observation that a certian riboflavin-requiring mutant of Bacillus subtilis excretes

Table 1. The Requirement for Pyridine Nucleotide in the Conversion of ADRAP to Riboflavin

Additions to or deletions from standard reaction mixture*	Riboflavin detected nmoles	µM
None	0.15	1.3
-ADRAP + 1 mM NADPH	0.17	1.4
+0.1 µM NADPH	0.22	1.9
+10 µM NADPH	0.38	3.2
+0.1 µM $NADP^+$	0.25	2.1
+10 µM $NADP^+$	0.58	4.9
+1.0 µM NADH	0.26	2.2
+10 µM NADH	0.38	3.2
+0.1 µM NAD^+	0.54	4.3
+10 µM NAD^+	0.65	5.1

* The standard reaction mixture is the one described in the text, containing Fractions A and B, but no pyridine nucleotide.

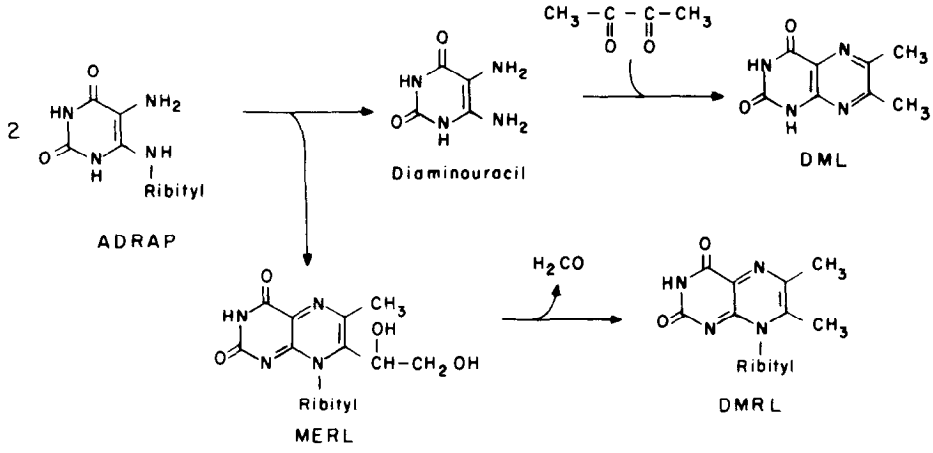

Fig. 3. Possible reactions involved in the conversion of ADRAP to DMRL.

a compound identified as 6-methyl-7-dihydroxyethyl-8-ribityllumazine (abbreviated as MERL). They suggest that MERL is an intermediate in the conversion of ADRAP to DMRL. The latter reaction would entail the removal of one carbon from MERL, probably as formaldehyde, to give DMRL. If these proposals are correct, the utilization of 2 moles of ADRAP to give one mole of MERL should also yield one mole of diaminouracil (as shown in Fig. 3). The latter compound is relatively unstable in aqueous solution, but it can be reacted with diacetyl to give the stable compound, 6,7-dimethyllumazine (DML, see Fig. 3). To test this possibility we added diacetyl (0.025 ml of a 5% solution) to an incubated reaction mixture, heated it at 70° for 20 minutes, and then analyzed the mixture for DML by HPLC. A fluorescent peak was detected which corresponded in elution pattern with standard DML. In a control mixture, not treated with diacetyl, this peak was absent. The amount of DML detected represented about 12% of the theoretical amount to be expected from the amount of DMRL and riboflavin produced. The low yield can be explained by the lability of diaminouracil.

Our conclusion that the source of the 4 carbons needed to convert ADRAP to DMRL is ADRAP is consistent with (a) the labeling evidence which suggests that these carbons are derived from an intermediate in the pentose phosphate cycle (8,9) and (b) the suggestion of Bresler et al. (7) that 2 moles

of ADRAP are used in the conversion and that MERL is an intermediate. The requirement for NAD^+ suggests that an oxidation might be required to generate MERL from 2 moles of ADRAP and that the resulting NADH is used in a subsequent reduction, perhaps in the conversion of MERL to DMRL.

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REFERENCES

1. Bacher, A. and Lingens, F. (1971) J. Biol. Chem., 246, 7018-7022.
2. Foor, F. and Brown, G.M. (1975) J. Biol. Chem., 250, 3545-3551.
3. Burrows, R.B. and Brown, G.M. (1978) J. Bacteriol., 136, 657-667.
4. Plaut, G.W.E. (1963) J. Biol. Chem., 238, 2225-2243.
5. Hollander, I. and Brown, G.M. (1979) Biochem. Biophy. Res. Communs., 89, 759-763.
6. Maley, G.F. and Plaut, G.W.E. (1959) J. Biol. Chem., 234, 641-647.
7. Bresler, S.E., Perumov, D.A., Chernik, T.P. and Glazunov, E.A. (1976) Genetika, 12, 83-91.
8. Ali, S.N. and Al-Khalidi, U.A.S. (1966) Biochem. J., 98, 182-188.
9. Alworth, W.L., Dove, M.F. and Baker, H.N. (1977) Biochemistry, 16,526-531