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Presence in *Escherichia coli* of a Deaminase and a Reductase Involved in Biosynthesis of Riboflavin

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Two enzymes have been partially purified from extracts of *Escherichia coli* B which together catalyze the conversion of the product of the action of GTP cyclohydrolase II, 2,5-diamino-6-oxy-4-(5'-phosphoribosylamino)pyrimidine, to 5-amino-2,6-dioxy-4-(5'-phosphoribitylamino)pyrimidine. These two compounds are currently thought to be intermediates in the biosynthesis of riboflavin. The enzymatic conversion occurs in two steps. The product of the action of GTP cyclohydrolase II first undergoes hydrolytic deamination at carbon 2 of the ring, followed by reduction of the ribosylamino group to a ribitylamino group. The enzyme which catalyzes the first step, herein called the "deaminase," has been purified 200-fold. The activity was assayed by detecting the conversion of the product of the reaction catalyzed by GTP cyclohydrolase II to a compound which reacts with butanedione to form 6,7-dimethyllumazine. The enzyme has a molecular weight of approximately 80,000 and a pH optimum of 9.1. The dephosphorylated form of the substrate is not deaminated in the presence of the enzyme. The assay for the enzyme which catalyzes the second step, referred to here as the "reductase," involves the detection of the conversion of the product of the deaminase-catalyzed reaction to a compound which, after treatment with alkaline phosphatase, reacts with butanedione to form 6,7-dimethyl-8-ribityllumazine. The reductase has a molecular weight of approximately 40,000 and a pH optimum of 7.5. Like the deaminase, the reductase does not act on the dephosphorylated form of its substrate. Reduced nicotinamide adenine dinucleotide phosphate is required as a cofactor; reduced nicotinamide adenine dinucleotide can be used about 30% as well as the phosphate form. The activity of neither enzyme is inhibited by riboflavin, FMN, or flavine adenine dinucleotide.

It has been known for some time that the riboflavin biosynthetic pathway originates with a purine compound, and that the purine ring, with the exception of carbon 8, is incorporated intact into the isoalloxazine ring of riboflavin (see refs. 15 and 16 for reviews). If the release of carbon 8 is one of the early reactions in the pathway, the product of the reaction should be a 4,5-diaminopyrimidine. Evidence for the existence of these diaminopyrimidines as intermediates in riboflavin biosynthesis has been obtained by Bacher and co-workers (1, 2, 10, 14). They isolated six groups of noncomplementing riboflavin-requiring mutants of *Saccharomyces cerevisiae* and characterized the excretion products of each, and on the basis of this evidence they have proposed a pathway for the biosynthesis of riboflavin (2).

Aside from the work of a number of investigators on riboflavin synthetase, the last enzyme of the pathway (see ref. 16 for a review of this subject), nothing was known about the enzymes involved in the biosynthesis of riboflavin until Foor and Brown (7) reported on the purification of an enzyme from *Escherichia coli* which catalyzes the reaction whereby GTP is converted to inorganic pyrophosphate, formic acid (derived from carbon 8 of GTP), and 2,5-diamino-6-oxy-4-(5'-phosphoribosylamino)pyrimidine ("phosphoribosylaminopyrimidine" [PRP] for short). These authors suggested that this enzyme, named GTP cyclohydrolase II, catalyzes the first of several enzymatic reactions involved in the biosynthesis of riboflavin. Evidence that guanine, or a guanine nucleoside or nucleotide, is a precursor of riboflavin had previously been supplied by other workers (3, 4). We have been exploring the possible importance of GTP cyclohydrolase II in the biosynthesis of riboflavin by investigating the metabolism of the product (PRP) of its action in the presence of extracts of *E. coli*. In the present paper we report on the existence and some properties of two enzymes, a deaminase and a reductase, which are necessary for the formation of putative intermediates in the riboflavin biosynthetic pathway.

MATERIALS AND METHODS

General. The source of the enzymes studied in this work was *E. coli* B, grown in a rich medium and harvested during the log phase. These cells were supplied as a frozen paste by the Grain Processing Co. GTP cyclohydrolase II was purified by the method of Foor and Brown (7). [*U*-^{14}C]GTP (50 μCi/ml, 528 mCi/mmol) and [8-^{14}C]GTP (50 μCi/ml, 52 mCi/mmol) were obtained from Amersham-Searle. Whatman 3MM chromatography paper, as 46- by 57-cm sheets, was obtained from Fisher Scientific. Thin-layer chromatography was performed with Eastman Kodak 13255 cellulose TLC plates; solvents were reagent grade. Ultrogels AcA 44 and AcA 54, supplied by LKB, were used for gel filtration chromatography. ECTEOLA-cellulose was obtained from Gallard-Schlesinger. Tris(hydroxymethyl)aminomethane (Tris) base, quaternary aminoethyl-Sephadex (Q-50-120), diethylaminoethyl-Sephadex (A-50-120), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and bacterial alkaline phosphatase were supplied by Sigma Chemical Co. Phosphocellulose (Servacel P23, 0.71 meq/g) was obtained from Accurate Chemical and Scientific Co.; hydroxylapatite was obtained from Bio-Rad. Butanedione (Baker grade) was supplied by J. T. Baker Co. and was stored at 6°C until used. 2-Mercaptoethanol (98%) came from Eastman Kodak and was stored at −6°C; 1 M solutions were made up at weekly intervals and stored at 6°C until used. 6,7-Dimethyllumazine (DML) was obtained from Aldrich Chemical Co. and Scintiverse scintillation fluid was from Fisher Scientific. 6,7-Dimethyl-8-ribityllumazine (DMRL) was synthesized by the method of Maley and Plaut (13). 6,7-Dimethyl-8-ribitylpterin (DMRPt) was synthesized by the method of Davoll and Evans (6).

Rationale for enzyme assays. PRP and similar orthodiamino pyrimidine compounds that have been proposed as intermediates in the riboflavin biosynthetic pathway are quite unstable in aqueous solution, but they are known to react with compounds with vicinal dicarbonyl groups to form pteridines which are not only much more stable, but also are intensely fluorescent, a property which aids in the detection of these compounds. The reactivity with dicarbonyl compounds of these putative enzymatic intermediates has been useful in our efforts to devise feasible and convenient assays for the enzymes involved. If it is assumed that the riboflavin biosynthetic pathway resembles that thought to exist in *S. cerevisiae*, and that PRP is the first intermediate in this pathway, the possible reaction that PRP might be expected to undergo would be either reduction of the ribosyl group to ribityl or deamination at carbon 2 of the pyrimidine ring. Both of these reactions would be expected to be necessary for the conversion of PRP to riboflavin, but the question is which might occur first. 5-Amino-4-ribitylaminopyrimidines are known to react with butanedione to form 6,7-dimethyl-8-ribitylpteridines (6, 13), and Foor and Brown (7) have shown that PRP (which contains a ribosyl group) reacts with glyoxal to form pterin and ribose-5-phosphate. The relevant point is that a ribosyl group is lost during the reaction of *N*-ribosyl compounds with butanedione, whereas the ribityl group is retained during similar reactions involving *N*-ribityl compounds. Thus, the enzymatic reduction of the ribosyl group of PRP, followed by treatment with butanedione and phosphatase, would be expected to yield DMRPt. However, if deamination of PRP occurs without reduction, subsequent reaction with butanedione should yield DML. If PRP undergoes both deamination and reduction, the product of reaction with butanedione would be DMRL, after dephosphorylation. These reactions are outlined in Fig. 1. Each of the end products shown in Fig. 1 is derived from PRP through a unique set of reactions, and, thus, evidence for the formation of one of these products from PRP would be evidence for the occurrence of the reactions in that particular set.

Enzymatic production of PRP. For each reaction mixture devised to measure deaminase or reductase activity, or both, radioactive substrate (PRP) was first generated from radioactive GTP through the action of GTP cyclohydrolase II. For this purpose, a reaction mixture was prepared to contain (per 0.3-ml final volume): 50 mM Tris-hydrochloride (pH 8.0), 2 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 65 μM [*U*-^{14}C]GTP (1.6×10^6 cpm), and 1.2 mU of GTP cyclohydrolase II. The mixture was incubated for 20 min at 39°C. After incubation, enough 100 mM ethylenediaminetetraacetic acid (pH 8.0) and 1 M 2-mercaptoethanol was added to make the solution 25 mM ethylenediaminetetraacetic acid and 50 mM 2-mercaptoethanol. Under these conditions the GTP is converted almost quantitatively to PRP.

Deaminase assay. Since evidence to be presented in the Results section indicates that enzymatic deamination of PRP (to produce compound II, Fig. 1) precedes reduction in the biosynthetic pathway, the following procedure was devised for the measurement of the deamination through the formation of DML via a set of reactions given in Fig. 1. The material to be assayed for activity (0.1 ml or less) was incubated with 0.028 ml of the reaction mixture described above for the preparation of PRP. Incubation was for 30 min at 39°C. To convert the enzymatic product to DML (see Fig. 1), butanedione (0.028 ml of a 1.2 M solution) was added to the incubated reaction mixture, and the solution was heated at 70°C for 20 min. Standard DML (5 nmol) was then added, and any precipitate that may have been formed was removed by filtration through a small piece of yarn. The clear solution was then applied quantitatively to a 3- by 46-cm strip of Whatman 3MM paper, and the material was subjected to chromatography with 3% NH_4Cl solution as the solvent. The position of migration of DML was located as a fluorescent zone by examination of the developed chromatogram under UV light. That portion of the paper strip containing the DML was cut from the strip and analyzed for radioactivity in a scintillation counter with the use of 5 ml of Scintiverse scintillation fluid. A radioactive scan of a developed chromatogram, shown in Fig. 2, illustrates that by this procedure radioactive DML produced by the combination of enzymatic deamination and reaction with butanedione can be separated from the other radioactive components of the reaction mixture. This observation confirms the feasibility of the assay. With the use of this assay we have shown a linear relationship between the amount of enzyme preparation added and the amount of DML detected. A unit of deaminase activity is

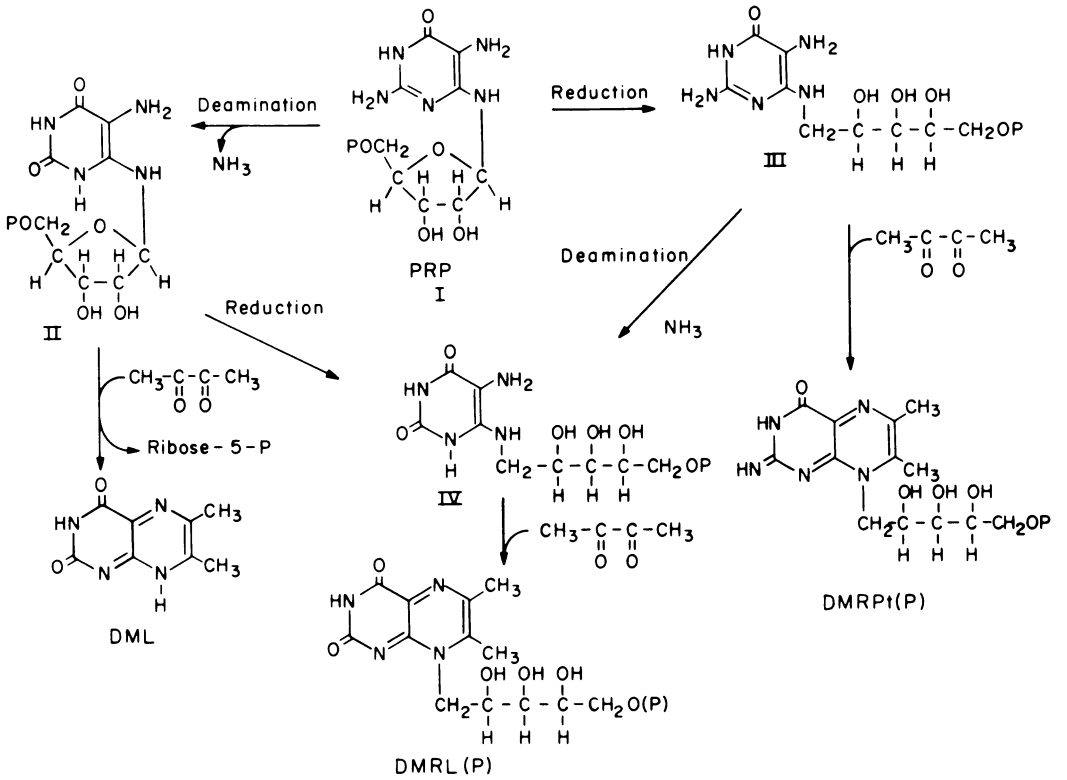

FIG. 1. *Possible enzymatic reactions that PRP can undergo and the nonenzymatic conversion of the resulting products to derivatives produced in the presence of butanedione. The phosphate groups in DMRL and DMRPt are shown in parentheses to indicate that the products detected by analyses have been dephosphorylated through the action of phosphatase. The possible enzymatic products are designated (as shown), and will be referred to hereafter in this paper, by Roman numerals as follows: I, 2,5-diamino-6-oxy-4-(5'-ribiosylamino)pyrimidine, or PRP for short; II, 5-amino-2,6-dioxy-4-(5'-phosphoribosylamino)pyrimidine; III, 2,5-diamino-6-oxy-4-(5'-ribitylamino)pyrimidine; and IV, 5-amino-2,6-dioxy-4-(5'-phosphoribitylamino)pyrimidine.*

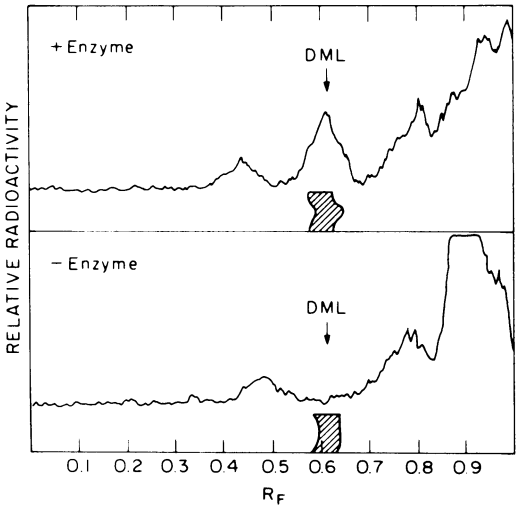

FIG. 2. *Separation by paper chromatography of radioactive DML from other radioactive components of reaction mixtures during assay for deaminase activity. Reaction mixtures (with and without a source of enzyme) were prepared and processed as described in the text. The figure shows the radioactive pattern obtained by analyses of the developed paper chromatograms for radioactivity with a strip scanner. The cross-hatched areas represent the zones of migration of standard DML, detected as fluorescent zones by examination under UV light.*

defined as the amount of enzyme needed for the production of 1 μmol of DML, the derivative of the enzymatic product (II, Fig. 1), per min under the conditions described above.

Reductase assay. Since the substrate for the reductase is the product (II, Fig. 1) of the action of the deaminase (see the Results section), this product must first be generated to assay for the reductase. For this purpose, radioactive PRP was generated from [*U*-^{14}C]GTP, as described above, followed by the addition of enough deaminase preparation to give 6 mU/ml. The mixture was then incubated for an additional 15 min to allow deamination to occur. The mixture was

made 10 mM in NADPH, and a portion (0.028 ml) of this mixture was incubated with no more than 0.1 ml of material to be assayed for reductase activity. This incubation was for 30 min at 39°C. A portion (0.028 ml) of a solution containing 50 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, and 6 U of alkaline phosphatase per ml was then added to each mixture, and incubation was continued for an additional 5 min to dephosphorylate components of the reaction mixture. To this incubated mixture was added butanedione (0.028 ml of a 1.2 M solution), and the material was heated at 70°C for 20 min to promote the formation of DMRL. Standard DMRL (2 nmol) was then added, and the resulting solution was applied quantitatively to a 3- by 45-cm strip of Whatman 3MM paper. The material was subjected to chromatography by development with *t*-butyl alcohol–water (60:40 by volume). The position of migration of the standard DMRL was detected as a fluorescent zone by examination under UV light. That portion of the chromatogram containing the DMRL was cut from the strip and analyzed for radioactivity in a scintillation counter in the presence of 5 ml of Scintiverse scintillation fluid. The separation of DMRL from other radioactive components of the reaction mixture is illustrated by the radioactive scan of a paper chromatogram shown in Fig. 3.

Preparation of crude extract. Frozen cells of *E. coli* B (as a cell paste) were ruptured in a Hughes press. The resulting broken-cell preparation was homogenized in 2 volumes of buffer containing 50 mM Tris-hydrochloride (pH 8.0), 1 mM ethylenediaminetetraacetic acid, and 0.05 mg of deoxyribonuclease I per ml. The deoxyribonuclease was added to degrade DNA to reduce the viscosity of the solution. The insoluble material was removed by centrifugation at 35,000 × *g* for 45 min. The resulting supernatant fluid was saved and will be referred to hereafter as the "crude extract." The protein concentration of such extracts was usually 30 mg/ml.

Protein determination. Protein concentration was measured by the method of Lowry et al. (11).

Miscellaneous methods. UV and visible light absorbances were determined with a Zeiss PMQ II spectrophotometer. Spectra in the UV region were recorded with a Perkin-Elmer model 202 recording spectrophotometer. Radiochromatograms were scanned with a Packard model 7200 radiochromatogram scanner. Radioactivity was determined with a Packard model 3320 Tri-Carb scintillation spectrometer. Reductions in volume of enzyme preparations were accomplished by ultrafiltration with the use of PM-10 filters.

RESULTS

Initial experiments. One of the first experiments we performed was to incubate dialyzed crude extract with ^{14}C-labeled PRP in the presence and absence of NADPH, after which the incubated reaction mixtures were treated with butanedione and alkaline phosphatase. Small amounts of DMRPt, DMRL, and DML were added as chromatographic standards, and the reaction mixtures were subjected to paper chromatography followed by two-dimensional thin-layer chromatography. The resulting chromatograms were then subjected to autoradiography to determine if any radioactive products corresponded to the chromatographic properties of the fluorescent standards. The details of this experiment were as follows. Crude extract was dialyzed overnight against 1,500 volumes of Tris-hydrochloride buffer (pH 8.0), and the dialyzed material (0.1 ml per reaction mixture) was used as a source of enzymes to prepare reaction mixtures (with and without the addition of 10 mM NADPH) as described in Materials and Methods. Three reaction mixtures were prepared to contain NADPH and two without NADPH. After incubation (see Materials and Methods for details), authentic DMRPt, DMRL, and DML (4 nmol each) were added (each to an individual reaction mixture) to the three mixtures containing NADPH; DML and DMRL were added to the two mixtures containing no NADPH. These

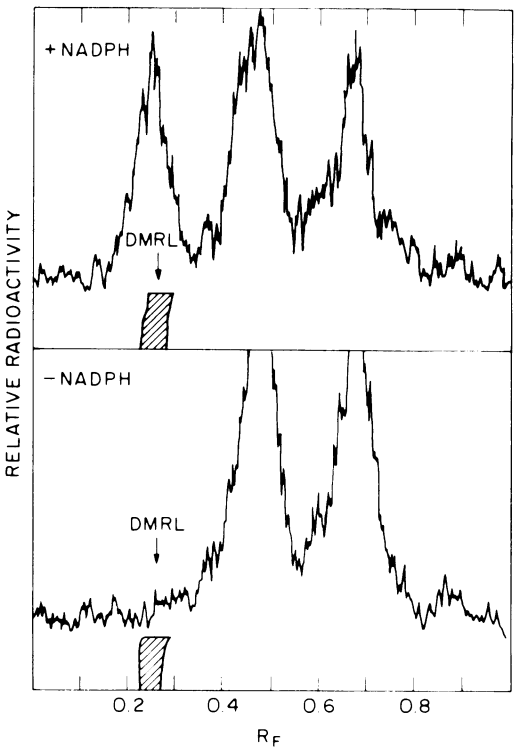

FIG. 3. *Separation of DMRL from other radioactive components of reaction mixtures during assay for reductase activity. Reaction mixtures were prepared (with and without NADPH) and processed as described in the text. The figure shows the radioactive pattern obtained by analyses of the developed paper chromatograms for radioactivity in a strip scanner. The cross-hatched areas represent zones of migration of standard DMRL, detected as fluorescent zones by examination under UV light.*

substances were added as chromatographic standards to visualize the zones of migration of these substances by the detection of fluorescence under UV light. The five reaction mixtures are labeled (in Fig. 4) as A, B, C, D, and E and differ (as shown in the figure) by (i) whether or not NADPH was present during incubation, and (ii) which standard was added. After the reaction mixtures were treated with alkaline phosphatase and butanedione, the mixtures were subjected to paper chromatography on strips (5 by 57 cm) of Whatman 3MM paper with *n*-butyl alcohol–pyridine–water (4:3:7 by volume) as the solvent system. The fluorescent zone corresponding to the standard was cut from each strip, the material was eluted from each (with water), and the resulting eluates were evaporated to dryness under reduced pressure. Each residue was dissolved in 0.05 ml of water, and 0.02-ml portions were subjected to thin-layer chromatography on individual cellulose plates (as described in Fig. 4). The results (Fig. 4) indicate that in the absence of NADPH radioactive DML was produced, but no DMRL was observed (Fig. 4B and C). However, when NADPH was added to the incubation mixtures, less radioactive DML was produced (Fig. 4D), and the production of DMRL in significant amounts was observed (Fig. 4E). The formation of radioactive DMRPt was not observed (Fig. 4A). Also, none of these products was detected in control reaction mixtures which contained heated crude extract in place of crude extract (data not shown).

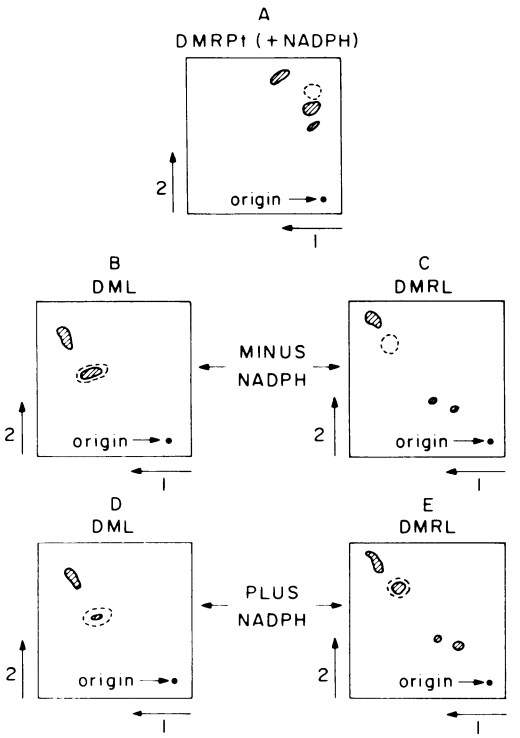

FIG. 4. *Conversion of radioactive PRP to end products (see Fig. 1) by incubation with dialyzed crude extract followed by treatment with alkaline phosphatase and butanedione. Details of the preparation and processing of reaction mixtures are given in the text. The materials (see the text) were subjected to thin-layer chromatography as follows: the plate containing material from reaction mixture A was developed with n-butyl alcohol–ethyl alcohol–water (50:15:35 by volume) in one dimension, followed by 3% NH_4Cl in the second dimension; plates containing material from reaction mixtures B, C, D, and E were all developed with 20% ethanol in the first dimension, followed by 3% NH_4Cl in the second dimension. After development, zones of migration of the standard compounds were detected by examination under UV light and are shown as dotted lines. Radioactive areas on the plates were detected by autoradiography and are shown as cross-hatched areas. The radioactive compound (or compounds) shown in the upper left corner of B, C, D, and E was not identified. It probably consists of unknown contaminating degradation products.*

From a consideration of these preliminary results in the light of the reactions outlined in Fig. 1, one can conclude that: (i) enzymes that catalyze deamination and reduction of PRP were present in the extract; (ii) NADPH is needed for reducing power to convert the ribose group to a ribityl group; and (iii) enzymatic deamination of PRP can occur without prior reduction of the ribose group. Thus, it appears that in *E. coli* either deamination occurs before reduction, or the deaminase can use either PRP or compound III as substrate. Further work (with the use of partially purified enzyme preparations) to be reported below indicates that the former is correct; i.e., in *E. coli* the enzymatic reaction sequence is deamination followed by reduction.

Purification of deaminase. The crude extract was subjected to filtration on an Ultrogel AcA 44 column as described in the legend to Fig. 5. The results (Fig. 5) indicate that the deaminase can be separated from the reductase by this procedure. Those fractions (64 to 76) containing the reductase activity were combined, and the material was subjected to further purification as described below. The fractions (50 to 62) containing deaminase activity were combined, and enough solid ammonium sulfate was added to make the solution 60% saturated with the salt (100% saturation = 3.9 M). The resulting precipitate was collected by centrifugation and dissolved in 12 ml of buffer (pH 8.0) containing 25 mM Tris-hydrochloride and 150 mM KCl. The solution was dialyzed overnight against 2 liters of the same buffer. The dialyzed deaminase preparation was applied to a column (2.1 by 12

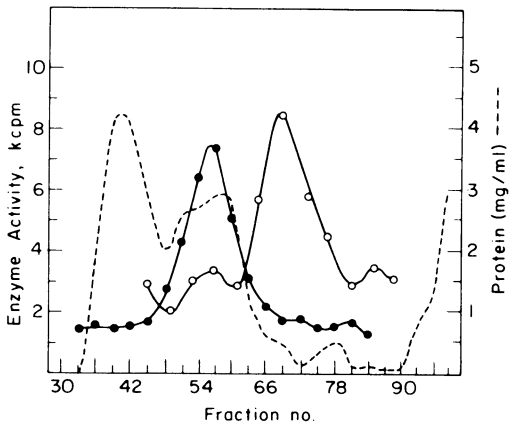

FIG. 5. *Fractionation of crude extract on a column of Ultrogel AcA 44. Crude extract (60 ml, 1.8 g of protein) was applied to a column (4.7 by 94 cm) of Ultrogel AcA 44 which had been equilibrated with buffer (pH 8.0) containing 50 mM Tris-hydrochloride and 0.01% Triton X-100. The column was developed with the same buffer, and fractions (15 ml each) were collected at a rate of 55 ml/h. Deaminase activity (●) was detected with the use of PRP as substrate. Reductase activity (○) could be determined only when deaminase (supplied from the deaminase-containing fractions from the column) was also present in the reaction mixtures. (-----) Protein concentration. Enzyme activity refers to the amount of radioactive DML (deaminase) or DMRL (reductase) produced. Radioactivity is expressed as kilocounts per minute (kcpm).*

cm) of quaternary aminoethyl-Sephadex which had been equilibrated with 25 mM Tris-hydrochloride buffer containing 150 mM KCl. The column was developed with 100 ml of the same buffer, followed by 400 ml of a linear gradient (0.15 to 0.6 M) of KCl in the buffer. Fractions of 6.1 ml each were collected at a rate of 10 fractions per h and assayed for deaminase activity as described in Materials and Methods. The activity eluted in a broad peak that extended from the beginning of the gradient to the major protein peak, which eluted at 0.3 M KCl. The fractions containing activity were combined (150 ml), concentrated by ultrafiltration to 20 ml, and dialyzed overnight against 2 liters of 5 mM potassium phosphate (pH 7.0).

The concentrated, dialyzed deaminase preparation from the quaternary aminoethyl-Sephadex column was applied to a phosphocellulose column (3.6 by 25 cm) which had been equilibrated with 5 mM potassium phosphate (pH 7.0). The column was developed with 2 liters of a linear gradient (0 to 0.5 M) of KCl in the same buffer. Fractions of 20 ml each were collected at the rate of 7 fractions per h. The deaminase activity eluted in a peak centered around 0.17 M KCl. These fractions were combined (440 ml) and concentrated to 2 ml by ultrafiltration. The concentrate was dialyzed overnight against 500 ml of 5 mM potassium phosphate buffer (pH 7.0).

The material from the previous step was applied to a hydroxylapatite column (1 by 10 cm) which had been equilibrated with 5 mM phosphate buffer (pH 7.0). The column was developed with 11 ml of the same buffer, followed by 80 ml of a linear gradient (5 to 250 mM) of potassium phosphate (pH 7.0). Fractions of 1.1 ml each were collected at a rate of 4 fractions per h. A 0.01-ml portion of each fraction to be assayed was mixed with 0.1 ml of 50 mM Tris-hydrochloride (pH 8.0). The resulting solutions were assayed for deaminase activity. The activity eluted in a peak centered around 75 mM phosphate. These fractions were combined (14 ml) and concentrated to 1 ml by ultrafiltration. The concentrate was dialyzed overnight against 500 ml of 50 mM Tris-hydrochloride buffer (pH 8.0). The dialyzed material was stored in 0.2-ml amounts at −6°C.

The above procedure resulted in a 200-fold increase in the specific activity of the deaminase with an overall yield of 18%.

Properties of deaminase. The deaminase has a molecular weight of approximately 80,000 as determined by filtration on a calibrated column of Ultrogel AcA 44. The pH optimum for the reaction is 9.2.

The following compounds were found to be ineffective as inhibitors of the reaction catalyzed by the deaminase: riboflavin, FMN, flavine adenine dinucleotide, GTP, GMP, AMP, CTP, and 6-hydroxy-2,4,5-triaminopyrimidine. Ethylenediaminetetraacetic acid stimulated the formation of product, but this is not surprising since divalent metal ions are known to contribute to the instability of PRP.

The enzyme was routinely stored at −6°C in 50 mM Tris-hydrochloride buffer (pH 8.0). The activity seems stable for at least a month under these conditions.

Substrate for the deaminase. A pertinent question is whether the deaminase acts only on PRP or whether it will use the corresponding ribityl compound (III, Fig. 1) as substrate. Since results presented later in this paper show that the reductase functions only after deamination has occurred, the conclusion to be drawn is that in *E. coli* compound III probably never occurs. Thus, whether or not the *E. coli* deaminase will use III as substrate cannot be tested since we have had no source of the ribityl compound.

Another question is whether or not the phosphate group of PRP is essential for the com-

pound to be a substrate for the deaminase. To answer this question, we dephosphorylated PRP by incubation with alkaline phosphatase (at pH 8.0), and then the resulting product was incubated with the deaminase. Since no DML was detected in this experiment, we can conclude that dephosphorylated PRP is not a substrate.

Product of the action of the deaminase. To show that the radioactive product of the action of the deaminase (after treatment with butanedione) is DML, a reaction mixture was scaled up to 3 ml, and after incubation the material was treated with butanedione in the normal fashion. The resulting fluorescent material was purified as described in the legend of Fig. 6. The UV absorption spectra of this purified material in water and in alkali compare favorably with those of authentic DML (see Fig. 6). The purified material was also shown to behave identically with authentic DML upon being subjected to thin-layer chromatography in 10 different solvent systems. Zones of migration of the enzymatically produced material were detected by autoradiography. R_f values of DML with these solvent systems are given in Table 1.

Finally, a portion of the purified material was analyzed for radioactivity, and its concentration was determined from its absorbance at 330 nm. From these values the specific radioactivity of the purified material was calculated to be 2.65×10^3 cpm/nmol. The theoretical specific activity to be expected after the conversion of the [U-^{14}C]GTP (with a specific activity of 6.8×10^3 cpm/nmol) to DML is 2.72×10^3 cpm/nmol. Thus, the similarity of the specific activity of the purified material to the theoretical value confirms the conclusion that the radioactive product is DML.

Purification of reductase. The fractions (64 to 76) containing the reductase activity from the Ultrogel AcA 44 column (see Fig. 5) were combined and reduced in volume to 5 ml by ultrafiltration. This concentrated material was applied to a column (1.8 by 118 cm) of Ultrogel AcA 54 which had been equilibrated with 50 mM Tris-hydrochloride buffer (pH 8.0). The column was developed with the same buffer. Fractions of 3.4 ml each were collected at a rate of 5 fractions per h. Reductase activity was eluted between 190 and 210 ml of eluent. This material was combined and concentrated to 2 ml by ultrafiltration, followed by dialysis overnight against 500 ml of buffer (pH 8.0) containing 25 mM Tris-hydrochloride and 50 mM KCl.

The dialyzed material from the previous step was applied to a diethylaminoethyl-Sephadex column (1 by 10 cm) which had been equilibrated with 25 mM Tris-hydrochloride buffer (pH 8.0) containing 50 mM KCl. The column was developed first with 15 ml of the same buffer, followed by 80 ml of a linear gradient (0.15 to 0.50 M) of KCl in 25 mM Tris-hydrochloride at pH 8.0. Fractions of 1.1 ml each were collected at a rate of 6 fractions per h. Reductase activity was eluted in a peak centered around

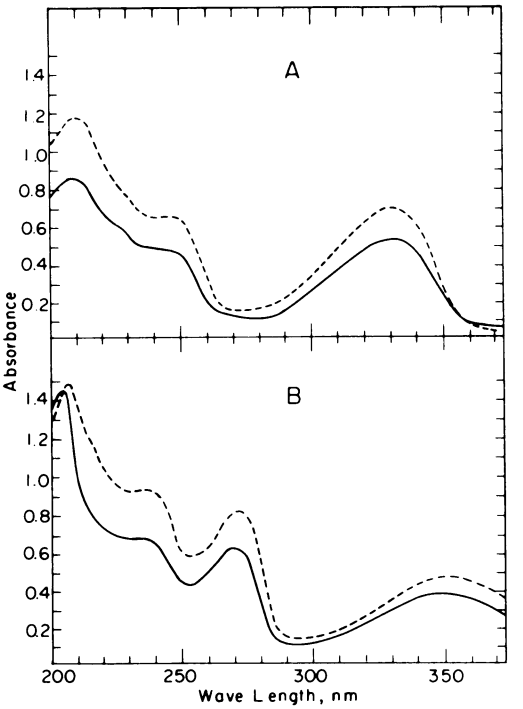

FIG. 6. *Absorption spectra of authentic DML* (——) *and the compound produced by reaction with butanedione of the product of the action of the deaminase* (-----). *(A) Spectra taken in water; (B) spectra taken in 1% ammonia. A typical reaction mixture (containing purified deaminase) for assay for deaminase activity was scaled up to 3 ml of total volume. Incubation and processing of the mixture was as described in the text for a typical reaction mixture. After the treatment with butanedione to produce DML, the mixture was filtered through glass wool to remove insoluble material, and the filtrate was concentrated to 1 ml under reduced pressure. This concentrated material was applied to a phosphocellulose column (1.1 by 56 cm), and the column was developed with water. Fractions of 4.9 ml each were collected at a rate of 4 fractions per h. The fractions (11 and 12) containing green fluorescent material when examined under UV light were combined and concentrated to 1 ml under reduced pressure. The concentrated material was applied to a column (1.1 by 55 cm) of ECTEOLA-cellulose, and the column was developed with water. Fractions of 5 ml each were collected at a rate of 4 fractions per h. The fractions (12 and 13) containing green fluorescent material were combined and concentrated to 0.95 ml under reduced pressure, and spectra were taken of this material in water and 1% NH_3.*

TABLE 1. *R_f values of DML and DMRL observed during thin-layer chromatography*

Solvent system[a]	R_f value	
	DML	DMRL
I	0.55	0.70
II	0.61	0.29
III	0.61	0.26
IV	0.57	0.59
V	0.81	0.70
VI	0.44	0.60
VII	0.56	0.41
VIII	0.65	0.25
IX	0.63	0.66
X	0.54	
XI		0.69

[a] Compositions of solvent systems were as follows: I, 3% aqueous NH_4Cl (wt/vol); II, *n*-butyl alcohol–ethyl alcohol–water (50:15:35 by volume); III, *n*-butyl alcohol–acetic acid–water (4:1:5 by volume); IV, *n*-propyl alcohol–1% aqueous NH_3 (2:1 by volume); V, *n*-butyl alcohol–pyridine–water (4:3:7 by volume); VI, 5% Na_2HPO_4 (wt/vol); VII, *t*-butyl alcohol–water (60:40 by volume); VIII, *t*-butyl alcohol–pyridine–water (60:15:25 by volume); IX, *t*-butyl alcohol–28% NH_3–water (60:5:35 by volume); X, *n*-propyl alcohol–ethyl acetate–water (7:1:2 by volume); and XI, 1620% ethyl alcohol.

0.25 M KCl. The fractions containing activity were combined (10 ml, total volume) and reduced in volume to 2 ml by ultrafiltration. The concentrated material was dialyzed overnight against 500 ml of 5 mM potassium phosphate buffer (pH 7.0).

The dialyzed solution from the previous step was applied to a column (1 by 10 cm) of hydroxylapatite previously equilibrated with 5 mM potassium phosphate buffer at pH 7.0. The column was developed first with 10 ml of the same buffer, followed by 80 ml of a linear gradient (5 to 300 mM) of potassium phosphate at pH 7.0. Fractions of 1 ml each were collected at a rate of 5 per h. Reductase activity was eluted with approximately 50 mM phosphate. The fractions containing the activity were combined (9 ml) and reduced in volume to 1 ml by ultrafiltration. This material was dialyzed overnight against 500 ml of 5 mM Tris-hydrochloride buffer (pH 8.0), and the dialyzed material was stored in 0.2-ml amounts at −6°C until used.

The purification scheme resulted in an overall increase of 200-fold in the specific activity of the reductase, with an overall yield of approximately 20%.

Properties of the reductase. The molecular weight of the reductase has been estimated at 37,000 by filtration through a calibrated column of Ultrogel AcA 54. The pH optimum of the enzyme is 7.5. No reaction occurs in the absence of NADPH. Reduced nicotinamide adenine dinucleotide is used about 30% as effectively as NADPH. The apparent K_m for NADPH (with a nonsaturating concentration, 12 μM, of compound II as substrate) was estimated at 5 μM from an Eadie-Hofstee plot. The purified enzyme can be stored at −6°C in 50 mM Tris-hydrochloride buffer (pH 8.0) for at least 1 month with no loss of activity.

Substrate for reductase. With the availability of purified reductase free from deaminase activity, it was possible to determine whether deamination is a necessary process before reduction can take place. For this purpose, the purified reductase was incubated with radioactive PRP in the presence of NADPH. The reaction mixture was then treated with butanedione and alkaline phosphatase and, after the addition of DMRPt as a standard, the material was first subjected to paper chromatography (*t*-butyl alcohol–water, 60:40 by volume) to separate any DMRPt that might have been formed from radioactive contaminants, followed by two-dimensional thin-layer chromatography (*n*-propyl alcohol–1% NH_3, 2:1 by volume; followed by 3% NH_4Cl in the second dimension) on 10- by 10-cm cellulose plates. The area corresponding to DMRPt (located on the plates as a fluorescent zone under UV light) was scraped from the plate and analyzed for radioactivity in a scintillation counter. No radioactive DMRPt could be detected. On the other hand, when the experiment was repeated in the presence of the purified deaminase, the expected amount of radioactive DMRL (equal to about 2% of the radioactivity added as GTP) was detected. These observations indicate that the substrate for the reductase is compound II (Fig. 1). Similar experiments have shown that if treatment with alkaline phosphatase is interposed after incubation of PRP with the deaminase and then reductase is added, no reduction takes place. Thus, the reductase resembles the deaminase in that the presence of the 5′-phosphate group on the substrate is necessary.

Product of the action of the reductase. To provide evidence about the nature of the product, the following procedure was used to obtain enough of the product for spectrophotometric analysis. A typical reaction mixture for assay of reductase activity (purified reductase was used) was scaled up to 3 ml (total volume). Incubation and treatment with phosphatase and butanedione were as described for a typical reaction mixture in Materials and Methods. After the treatment with butanedione, the material was applied to a column (1 by 67 cm) of phosphocellulose. The column was developed at 4°C with water. Fractions of 0.8 ml each were collected at

a rate of 10 fractions per h. The fractions (67 to 77) containing green fluorescent material were combined and concentrated under reduced pressure to 0.5 ml. The concentrated solution was applied to a column (1 by 67 cm) of ECTEOLA-cellulose, and the column was developed with water at 4°C. Fractions of 0.8 ml each were collected at a rate of 10 fractions per h. The fractions (75 to 85) containing green fluorescent material were combined and concentrated under reduced pressure to 0.5 ml. This solution was applied to a strip (10 by 46 cm) of Whatman 3MM paper, and the material was subjected to chromatography with *t*-butyl alcohol-water (60:40 by volume) as the solvent. The green fluorescent band on the developed chromatogram was eluted from the paper with water. The eluate was evaporated to dryness under reduced pressure, and the residue was dissolved in 1.0 ml of 0.2% NH_3 (pH 11). After the spectrum was taken in alkali, the solution was adjusted to pH 1.0 by the addition of HCl and the spectrum was taken again. The yield of the purified fluorescent material was poor owing to the degradation of the material during the purification steps. The UV spectra of the purified material in acid and alkali (Fig. 7) are very similar to those of authentic DMRL (λ_{max} at 279 and 313 nm in alkali; λ_{max} at 256 and 407 nm in acid). Additional evidence that the purified substance is DMRL was obtained with the observation that it migrated identically with authentic DMRL upon being subjected to thin-layer chromatography in 10 different solvent systems (see Table 1 for R_f values for DMRL).

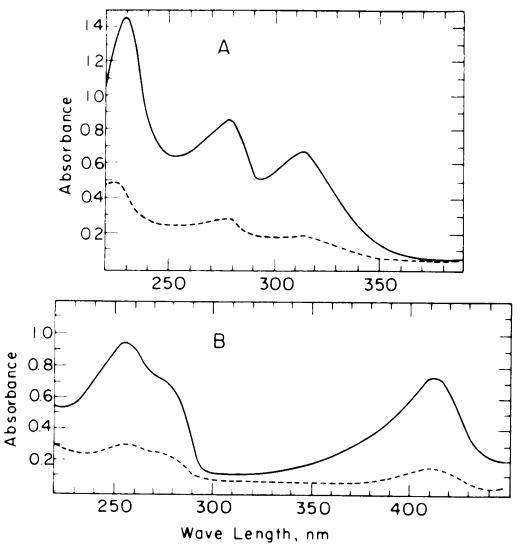

FIG. 7. *Absorption spectra of authentic DMRL* (——) *and the compound produced by reaction with butanedione of the dephosphorylated product of the action of the reductase* (-----). *(A) Spectra taken in 0.2% NH_3 (approximately pH 11); (B) spectra taken at pH 1.0.*

If enzymatic reduction occurs without dephosphorylation, treatment of the enzymatic product with butanedione should yield DMRL-5′-phosphate instead of DMRL. However, since DMRL-5′-phosphate has not been available to us as a chromatographic standard, treatment of the enzymatic product with alkaline phosphatase has been a routine procedure in the assay so that the properties of the product can be compared directly with those of the standard, DMRL. To obtain evidence bearing on whether or not the phosphate group is retained during the action of the reductase, material produced by the action of the reductase (i.e., a typical incubated reaction mixture to which was added initially 1.6×10^5 cpm of radioactive GTP) was divided into two equal portions. Only one of the two portions was treated with alkaline phosphatase in the usual fashion, and both were then subjected to analysis for radioactive DMRL by paper chromatography as described for the routine assay. In the sample that had been treated with phosphatase, radioactive DMRL in the amount of 12,000 cpm was detected, whereas the amount detected in the sample not treated with phosphatase was only 3,200 cpm. In the latter case, a radioactive scan of the paper chromatogram showed most of the radioactivity had remained at or near the origin. This material at the origin was eluted from the chromatogram with water, and the resulting eluate was divided into two equal portions. One of the two portions was treated with phosphatase, and the two were subjected to two-dimensional thin-layer chromatography. The solvent used in the first dimension was *t*-butyl alcohol-water (60:40 by volume), and 3% (wt/vol) NH_4Cl was used in the second dimension. The developed chromatograms were examined under UV light to locate the fluorescent zones of migration of the standard DMRL, and then they were subjected to autoradiography to detect radioactive zones. The results indicated that the material treated with phosphatase contained four radioactive products, one of which migrated identically with standard DMRL; the identities of the other radioactive products were not established. No DMRL was evident in the sample which was not treated with phosphatase. These observations are consistent with the notion that DMRL-P was a product formed in the absence of phosphatase treatment and, thus, that the phosphate group is retained during enzymatic reduction. The most likely explanation for the relatively small amount of DMRL that was observed on the paper chromatogram to be produced without

phosphate treatment is that one or more of the enzyme preparations used in the incubations were contaminated with phosphatases.

DISCUSSION

Because of their instability, the products of the action of the deaminase and the reductase could not be isolated in the quantities needed for direct characterizations. However, the indirect evidence presented in this paper leaves little doubt about the identity of the enzymatic products and thus also provides valuable information about the nature of the enzymatic reactions.

We have presented no direct evidence in this paper to show that the deaminase and the reductase are involved in the synthesis of riboflavin, although this possibility seems very likely in the light of what has been learned about riboflavin biosynthesis from other sources. This likelihood has received support from some recent preliminary observations in our laboratory that the product of the action of the reductase can be converted enzymatically to riboflavin in the presence of crude extracts of *E. coli* (I. Hollander, unpublished data). This system is currently under further investigation.

The results presented in this paper support the suggestion of Foor and Brown (7) that GTP cyclohydrolase II catalyzes the first of several reactions involved in the biosynthesis of riboflavin. The evidence suggests that the pathway shown in Fig. 8 is operative in *E. coli*. Reaction A is catalyzed by GTP cyclohydrolase II, reaction B by the deaminase, and reaction C by the reductase. Reaction E is catalyzed by riboflavin synthetase, an enzyme which has been studied intensively (see ref. 16 for a review). This reaction involves the utilization of 2 mol of DMRL, one as the donor of a 4-carbon unit and the second as the acceptor of the 4-carbon unit, to yield riboflavin and 5-amino-2,6-dioxy-4-ribitylaminopyrimidine.

Very little is known about the enzymology of reaction D. Since Lingens et al. (10) have presented evidence that in yeast two genes are involved in this transformation, one can suppose that more than one enzyme is involved in *E. coli*. The nature and source of the 4-carbon compound needed in the transformation is unclear, although some recent evidence from Bresler's laboratory (5) indicates that in *Bacillus subtilis* this material is supplied by the ribityl group of compound IV (Fig. 8). If this is true, then compound IV would function in a dual role in reaction D in that it would act both as a donor of the 4-carbon unit and as the acceptor of the 4-carbon unit. This suggestion is based on the identification of the excretion product of a riboflavin-requiring mutant of *B. subtilis* as a compound which one could visualize as being an intermediate in such a reaction. It should be mentioned, however, that this proposal is not consistent with the findings of Mailänder and Bacher (12), who reported that in *Salmonella typhimurium* the ribose group of guanine nucleotide is not incorporated into any portion of riboflavin except for the ribityl group. This issue will be settled only when the relevant enzyme work is done.

The results of the present paper are in general

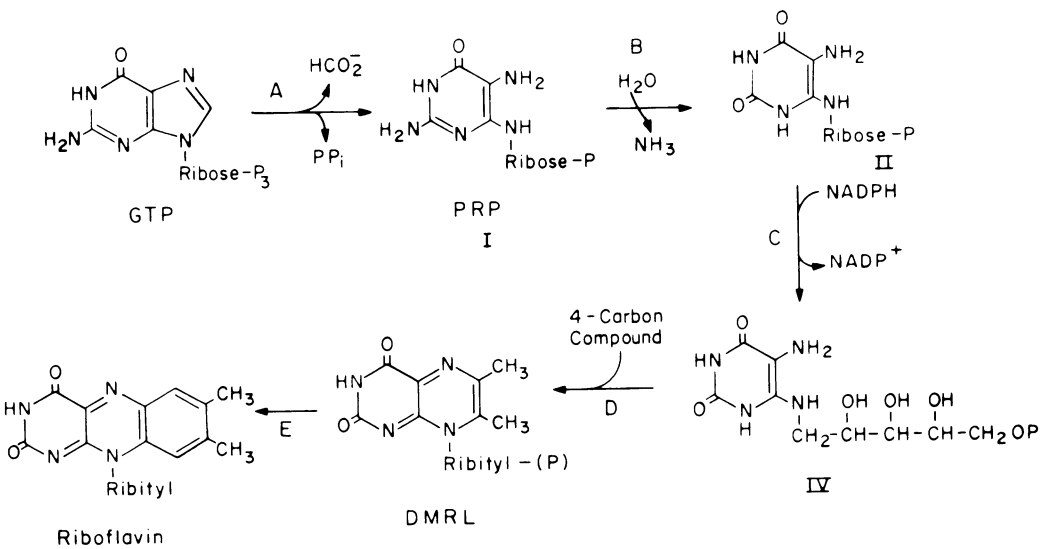

FIG. 8. *Probable pathway for the biosynthesis of riboflavin in E. coli. The phosphate group is shown in parentheses in compound V (DMRL) since it is not yet known whether or not the phosphate group is present in this intermediate.*

agreement with the conclusions drawn by Bacher and Lingens (2) about the pathway of riboflavin biosynthesis, based on evidence derived from excretion patterns of different classes of riboflavin-requiring mutants of *S. cerevisiae*. The major difference is that the genetic evidence indicates that in yeast reduction precedes deamination, whereas the enzymatic evidence indicates that in *E. coli* deamination occurs first. The genetic evidence provided no indication of whether or not the ribose group of guanine nucleotide is retained and reduced directly to the ribityl group of riboflavin, since the compounds that were identified contained no ribose groups (the ribosyl groups were presumably lost during reaction with butanedione). However, other evidence supplied by Mailänder and Bacher (12) indicated that in a guanosine-requiring mutant of *S. typhimurium* the ribose group of guanine nucleotide is retained and reduced directly to the ribityl group of riboflavin, an observation which is consistent with the conclusions drawn from the evidence presented in this paper. From the evidence reported by Bacher and co-workers and that presented in this paper, it seems likely that the enzymatic reactions proceed in a different order in the two systems (i.e., reduction followed by deamination in yeast, and the reverse order in *E. coli*).

Since we have found that during enzymatic deamination and reduction the phosphate group is retained in the intermediates, a question that arises is whether the phosphate group is retained throughout the whole enzymatic pathway to yield finally FMN rather than riboflavin. This possibility can be discounted, since in a recent paper Harzer et al. (9) reported that DMRL-5′-phosphate is not a substrate for riboflavin synthase from *E. coli* and *B. subtilis*. It is also likely that the same is true in yeast, since Harvey and Plaut (8) have found that yeast riboflavin synthase will bind riboflavin (the product) very effectively, but FMN only poorly, if at all. Thus, the evidence indicates that at some stage between the biosynthetic intermediate, 5-amino-2,6-dioxy-4-(5′-ribitylamino)pyrimidine, and riboflavin the phosphate group is removed enzymatically.

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