The xylene ring of riboflavin originates by dismutation of the precursor, 6,7-dimethyl-8-ribityllumazine. The formation of the latter compound requires a 4-carbon unit as the precursor of carbon atoms 6a, 6, 7, and 7a of the pyrazine ring. The formation of riboflavin from GTP and ribose phosphate by cell extract from Candida guilliermondii has been observed by Logvinenko et al. (Logvinenko, E. M., Shavlovsky, G. M., Zakal'sky, A. E., and Zakhodylo, I. V. (1982) Biokhimiya 47, 931-936). We have studied this enzyme reaction in closer detail using carbohydrate phosphates as substrates and synthetic 5-amino-6-ribitylamino-2,4-(1H,3H)-pyrimidinedione or its 5'-phosphate as cosubstrates. Several pentose phosphates and pentulose phosphates can serve as substrate for the formation of riboflavin with similar efficiency. The reaction requires Mg$^{2+}$. Various samples of ribulose phosphate labeled with 14C or 13C have been prepared and used as enzyme substrates. Radioactivity was efficiently incorporated into riboflavin from [1,14C]ribulose phosphate, [3,5-14C]ribulose phosphate, and [5-14C]ribulose phosphate, but not from [4-14C]ribulose phosphate. Label from [1-13C]ribose 5-phosphate was incorporated into C6 and C6a of riboflavin. [3,5,13C]Ribose 5-phosphate yielded riboflavin containing 13C atoms 1*-3* biosynthetically equivalent to C1-C3 of the pentose pool. (iii) C4* of riboflavin is equivalent to C5 of the pentose pool. (iv) Riboflavin in the enzymatic reaction. It follows that the 4-carbon unit used for the biosynthesis of 6,7-dimethyl-8-ribityllumazine consists of the pentose carbon atoms 1, 2, 3, and 5 in agreement with earlier in vitro studies.

The biosynthesis of riboflavin leads from GTP to 5-amino-6-ribitylamino-2,4-(1H,3H)-pyrimidinedione 5'-phosphate (Compound 1, Fig. 1) (3-6) which is converted to 6,7-dimethyl-8-ribityllumazine (Compound 2, Fig. 1) by the addition of a 4-carbon unit. Dismutation of 2 yields riboflavin and the pyridine 4 (for reviews see Refs. 7 and 8). The structure and origin of the 4-carbon unit required for the formation of 2 remained elusive for several decades in spite of considerable efforts. Early work by Plaut and Broberg (9, 10) showed that both terminal carbon atoms of glucose, i.e. C1 and C6, were incorporated into the xylene ring of riboflavin with similar efficiency (9, 10). Subsequent studies led to the suggestion of acetoin (11, 12), diacetyl (13), pyruvate (14), tetroses (15), pentoses (16, 17), and 5-amino-6-ribitylamino-2,4-(1H,3H)-pyrimidinedione (4) (18, 19) as the ultimate source of the 4-carbon moiety. Work by Alworth and co-workers (17) specifically suggested the incorporation of C1 of ribose into the benzenoid moieties of dimethylbenzimidazole and riboflavin.

We have previously studied the incorporation of a wide variety of 13C-labeled precursors into riboflavin by the flavinogenic fungus Ashbya gossypii (20-25). A large number of incorporation experiments with a variety of single and multiple 13C-labeled precursors has been performed and can be summarized as follows. (i) Diacetyl and other symmetrical molecules can be ruled out definitely as precursors. (ii) Carbon atoms 1*-3* are biosynthetically equivalent to C1-C3 of the pentose pool (see Fig. 1 for explanation of the arbitrary nomenclature used). (iii) C4* of riboflavin is equivalent to C5 of the pentose pool. (iv) C4 of the pentose pool has no equivalent in the 4-carbon moiety; it is eliminated during the biosynthetic process through a rearrangement of the carbon skeleton in which C5 and C6 are interconnected intramolecularly. Consequently, the carbon atoms 3 and 5 of the precursor ribose are contiguous in the heterocyclic moieties of the products 2 and 3.

Several attempts have been made to study the formation of the xylene ring in vivo. The enzymatic formation of riboflavin from 5-amino-6-ribitylamino-2,4-(1H,3H)-pyrimidinedione (4) and acetoin or pyruvate by cell extract of Eremothecium ashbyii has been reported (12, 14). Plaut has suggested that these results may be explained by the enzymatic formation of diacetyl which would subsequently react nonenzymatically with the pyridine 4 to yield the lumazine 2 (26).

More recently, Hollander et al. (19) reported on the formation of riboflavin from 1 or 4 by cell extracts of Escherichia coli. Surprisingly, this reaction required no second substrate. It was, therefore, proposed, in agreement with an earlier hypothesis by Breslet et al. (18), that 4 can donate its ribityl group as a 4-carbon precursor by a dismutation reaction which would be formally analogous to the formation of riboflavin from 2.
Enzymatic Formation of riboflavin by cell extract from C. guilliermondii

The reaction mixture contained 0.72 mM I, 38 mM Tris hydrochloride, pH 8.0, 3.8 mM MgCl₂, 10 mM dithioerythritol, 0.95 mM of the respective carbohydrate and 0.6 mg of protein; total volume, 400 µl. Incubation was at 37 °C for 1 h. Riboflavin was monitored by bioassay with Lactobacillus casei. Results are corrected for blank values (approximately 1.6 µM riboflavin).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Riboflavin formed µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Arabiose 5-phosphate</td>
<td>4.5</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.1</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Ribose</td>
<td>0</td>
</tr>
<tr>
<td>Ribose 5-phosphate</td>
<td>9.0</td>
</tr>
<tr>
<td>Ribulose</td>
<td>0</td>
</tr>
<tr>
<td>Ribulose 5-phosphate</td>
<td>5.4</td>
</tr>
<tr>
<td>Xylulose 5-phosphate</td>
<td>8.2</td>
</tr>
</tbody>
</table>

TABLE II

Enzymatic formation of riboflavin by cell extract from C. guilliermondii

Reaction mixtures contained 40 mM Tris hydrochloride, pH 8.0, 4 mM MgCl₂, 1 mM ribose phosphate, pyrimidine substrate as indicated, and protein. Results are corrected for blank values. For other details see Table I.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Riboflavin formed µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>1 (0.63 mM)</td>
<td>8.1</td>
</tr>
<tr>
<td>4 (0.44 mM)</td>
<td>5.3</td>
</tr>
</tbody>
</table>

TABLE III

Enzymatic formation of riboflavin by cell extract from C. guilliermondii

Reaction mixtures contained 30 mM Tris hydrochloride, pH 8.2, 2 mM MgCl₂, 1.5 mM 1, 5 mM ribose phosphate, and protein. Data are corrected for blank values. For other details see Table I.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Riboflavin formed %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>NADH (1 mM)</td>
<td>80</td>
</tr>
<tr>
<td>NAD (1 mM)</td>
<td>37</td>
</tr>
<tr>
<td>NADPH (1 mM)</td>
<td>83</td>
</tr>
<tr>
<td>NADP (1 mM)</td>
<td>34</td>
</tr>
<tr>
<td>ATP (2 mM)</td>
<td>96</td>
</tr>
<tr>
<td>EDTA (10 mM)</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

ATP (2 mM) and thiamine pyrophosphate. The reaction was completely inhibited by 10 mM EDTA (Table III). Cell extract dialyzed against buffer containing EDTA could be partially reactivated by the addition of an excess of Mg²⁺.

In order to study the fate of individual pentose carbon atoms in closer detail, we prepared enzymatically a variety of ribulose 5-phosphate samples specifically labeled with ¹³C. Commercial samples of radioactive glucose and glycerol served as precursors. 6-Phosphogluconate has a high retention time on the anion exchanger Dowex 1-X8 and, therefore, can be easily separated from the other intermediates occurring in the reaction sequence. We thus chose to isolate this intermediate in radiochemically pure form. It was subsequently converted to the desired product, ribulose 5-phosphate. It should be mentioned that radioactive ribulose 5-phosphate samples decomposed rapidly even at the temperature of liquid nitrogen.

EXPERIMENTAL PROCEDURES

We studied the enzymatic formation of riboflavin from synthetic pyrimidine substrates by cell extracts of C. guilliermondii. The reaction requires the addition of 4 carbon atoms. As shown in Table I, each of two pentose phosphates and two pentulose phosphates could serve as donor of the 4-carbon unit. The highest yield of riboflavin was obtained with ribose phosphate as substrate. Unphosphorylated pentoses and hexose phosphates could not serve as precursors.

The pyrimidine 4 and its 5'-phosphate 1 could both serve as precursors for the in vitro formation of the heterocyclic moiety of the vitamin (Table II). Yields of riboflavin were similar with both types of pyrimidine substrate.

Oxidized and reduced pyrimidine nucleotide cofactors (1 mM) did not increase the formation of riboflavin, nor did...
A loss of more than 50% usually occurred within several days. Throughout this study, the ribulose 5-phosphate samples were, therefore, prepared immediately prior to further use.

The radioiodinated ribulose 5-phosphate samples were used as substrates for the enzymatic formation of riboflavin with the pyrimidine phosphate as cosubstrate. Light riboflavin synthase from B. subtilis was added to the reaction mixtures to ensure quantitative conversion of the lumazine intermediate 2 to riboflavin. The vitamin was isolated and purified to constant specific radioactivity as described under "Experimental Procedures." It was subsequently converted to lumichrome (Compound 6, Fig. 2) by illumination with visible light, in order to establish the radioactivity distribution between the ring system and the side chain. Lumichrome was obtained in radiochemically pure form by high pressure liquid chromatography.

Radioisotope from [1-14C]ribulose 5-phosphate and [5-14C] ribulose 5-phosphate was incorporated into the isoalloxazine moiety of riboflavin with high and similar efficiency (Table IV). Specific activities of riboflavin and of lumichrome obtained by photochemical degradation (Fig. 2) showed no significant difference, thus indicating that no radioactivity was contributed to the riboflavin side chain. [3,5-14C]Ribulose 5-phosphate contributed label to riboflavin with about the same efficiency as [5-14C]ribulose 5-phosphate. This indicates that C3 and C5 of the precursor are both incorporated into the product; if C5, but not C3, were incorporated, one would expect that the incorporation rate from [3,5-14C]ribulose 5-phosphate should be only half as compared to [5-14C]ribose 5-phosphate. On the other hand, very little radioisotope was transferred to the product, riboflavin, from [4-14C]ribulose 5-phosphate. This finding is in excellent agreement with results of earlier in vivo studies which had indicated that all pentose carbon atoms except C4 contribute to the xylene ring of riboflavin (20-25).

For further confirmation of the correspondence between the in vitro system and the actual biosynthetic pathway, we decided to prepare two 14C-labeled samples of ribose 5-phosphate. The positions of the labels were chosen (a) to probe the origin of all four carbon atoms of the 4-carbon unit, (b) to probe for intact conversion of the pentose carbon chain by demonstrating intact incorporation of the C2-C3 bond, i.e. the bond which is cleaved in normal metabolism of the pentose phosphate carbon chain, and (c) to demonstrate the occurrence of the rearrangement reaction in which C4 is excised and C3 and C5 are intramolecularly reconnected. Consequently, one sample of the precursor was labeled with 14C at C1 and the other carried three intramolecular 13C labels, each at 99% enrichment, at C2, C3, and C5. Conversion of the latter precursor by the process demonstrated in vivo should give a sample of riboflavin in which each of the two 4-carbon units is contiguously labeled with 14C at C2*, C3*, and C4* (see Fig. 1 for the arbitrary notation used).

Both precursors were synthesized enzymatically on a gram scale from the appropriately labeled glucose 6-phosphate by the combined action of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase. Since ribose 5-phosphate has been found (Table I) to be a more efficient substrate for riboflavin formation than d-ribulose 5-phosphate, the initially formed d-ribulose 5-phosphate was equilibrated with phosphoriboisomerase, first at room temperature and then, in view of the temperature dependence of the equilibrium (47), at lower temperature, to give a mixture of predominantly d-ribose 5-phosphate and a smaller amount of d-ribulose 5-phosphate. This mixture was isolated and used directly, without separation of the two components, in the cell-free synthesis of riboflavin. The d-[2-13C]glucose 6-phosphate for the synthesis of [1-13C]ribose 5-phosphate was prepared by phosphorylation of commercially available d-[2,3,5-13C]glucose (99% 13C) with ATP and hexokinase. The overall yield, based on glucose, was 85%. The d-[3,5,13C3]ribulose 5-phosphate was obtained from d-[1,3,4,6-13C4]glucose 6-phosphate, which in turn was prepared from [1,3,15C]glycerol 3-phosphate by the action of glycerolphosphate dehydrogenase, triosephosphate isomerase, and aldolase, followed by fructose 1,6-diphosphatase and phosphoglucoisomerase. The glyceraldehyde 3-phosphate was prepared with ATP and glycerokinase from [1,3,15C]glycerol synthesized chemically (35, 36) from K13CN and [1-13C]acetic acid. It was essential for good yields in the synthesis of glucose 6-phosphate to isolate and purify the glyceraldehyde 3-phosphate, in order to remove ATP and ADP. The overall yield of [2,3,5,13C4]ribose 5-phosphate was about 30% based on glyceraldehyde 3-phosphate. Analysis of both labeled precursors by 13C-NMR spectroscopy confirmed the expected positions of the label.

The conversion of the two 13C-labeled precursors into riboflavin with the cell-free system from C. guillermondii was carried out under conditions carefully optimized for maximum yields of riboflavin. From incubations with 250 pmol of d-[1-13C]ribose 5-phosphate and 300 pmol of d-[2,3,5-13C]ribulose 5-phosphate, respectively, 8.24 and 3.99 pmol of pure riboflavin were obtained from [1,3,4,6-13C4]glucose 6-phosphate, which in turn was prepared from [1,3,15C]glycerol 3-phosphate by the action of glycerol dehydrogenase, triosephosphate isomerase, and aldolase, followed by fructose 1,6-diphosphatase and phosphoglucoisomerase. The glyceraldehyde 3-phosphate was prepared with ATP and glycerokinase from [1,3,15C]glycerol synthesized chemically (35, 36) from K13CN and [1-13C]acetic acid. It was essential for good yields in the synthesis of glucose 6-phosphate to isolate and purify the glyceraldehyde 3-phosphate, in order to remove ATP and ADP. The overall yield of [2,3,5,13C4]ribose 5-phosphate was about 30% based on glyceraldehyde 3-phosphate. Analysis of both labeled precursors by 13C-NMR spectroscopy confirmed the expected positions of the label.

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Enzymatic Formation of 6,7-Dimethyl-8-ribityllumazine

**FIG. 3.** $^{13}$C NMR spectrum of riboflavin formed in vitro from [1-$^{13}$C]ribose 5-phosphate.

vin were isolated. These two samples were subjected to analysis by $^{13}$C NMR spectroscopy. The spectrum of riboflavin from d-[1-$^{13}$C]ribose 5-phosphate is shown in Fig. 3. Absolute $^{13}$C enrichments were calculated using the average of the ribityl carbon signals as natural abundance standard (1.1%). The data (Scheme A) show high enrichment of the two 1* positions, i.e. carbon atoms 6 and 8α (see Fig. 2 for systematic nomenclature of carbon atoms), confirming that C1 of the pentose phosphate gives rise to C1* of the 4-carbon unit as demonstrated in vivo (23). A small amount of label seems to be present at C2*, i.e. carbon atoms 5α and 8 of riboflavin (see Fig. 1 and Scheme A for numbering). This, too, is consistent with observations made in the in vivo feeding experiments, which had shown some scrambling of label from C1 to C2 in the metabolism of ribose (23, 25).

The $^{13}$C NMR spectrum of riboflavin from d-[2,3,5-$^{13}$C$_3$]ribose 5-phosphate is shown in Fig. 4. The carbons of the two 4-carbon moieties show the expected enrichments (Scheme B). The rather complex $^{13}$C-$^{13}$C coupling patterns are most easily deduced for carbons which have the fewest enriched one-bond carbon neighbors. Thus, the optimal sites for examination of coupling are 8α (1*), 5α (2*), 9α (3*), and 7α (4*) (see Scheme C). The other four signals (6, 7, 8, and 9) yield entirely consistent information, but the patterns are somewhat more complicated. Carbon atoms 5α (2*) and 7α (4*) each show coupling to one other labeled carbon. Notably, 97% of the total signal due to 7α is one-bond carbon coupled. The 3* positions, i.e. carbons 7 and 9α, appear predominantly as doubly coupled species. In addition, a small amount of singly coupled species is discernible, reflecting again some metabolism of ribose 5-phosphate in the system (see below). Carbon atoms 8 and 9 show more complex patterns due to a combination of single coupling to the 3* position and statistical coupling to each other as a result of the combination of two highly enriched 4-carbon units. A small amount of scrambling of label into the 1* position, i.e. C6 and C8α, is also evident, with about 48% of the 8α signal showing statistical coupling to the 86% enriched C8. This presumably reflects the same process which leads to scrambling from C1 to C2 of the pentose. A small amount of labeling of the 1* position from d-[2,3-$^{13}$C$_2$]ribose has also been observed in the in vivo experiments. In summary, the data confirm that the pentose carbons 2, 3, and 5 correspond to C2*, C3*, C4* of the alloxazine moiety, with loss of the pentose C4.

Studies by Hollander et al. (19) had suggested that the xylene moiety of riboflavin can be formed from the ribityl side chain of the pyrimidine 4 by enzymes from E. coli. In order to check whether this reaction occurs in C. guilliermondii, we synthesized 5-amino-6-[1-$^{13}$C]ribitylamino-2,4(1H,3H)-pyrimidinedione. Treatment of this compound with cell extract of C. guilliermondii in the presence of unlabeled ribose 5-phosphate afforded riboflavin which was photochemically degraded to lumichrome (Compound 5, Fig. 2). The pyrimidine contributed its isotope to riboflavin with high efficiency. However, the lumichrome fragment was devoid of radioactivity (Table V). It follows that radioactivity from the purine precursor was exclusively incorporated into the ribityl side chain of the vitamin.

In a similar experiment, 5-amino-6-[1-$^{13}$C]ribitylamino-2,4(1H,3H)-pyrimidinedione was treated with cell extract of C. guilliermondii mutant B6 using unlabeled ribose 5-phosphate as second substrate. This mutant is devoid of riboflavin synthase. Hence, the experiment yields the riboflavin precursor, 6,7-dimethyl-8-ribityllumazine (2). Photochemical degradation yielded 6,7-dimethylllumazine (Compound 6, Fig. 2). As in the previous experiment, the radioactive label was
**Enzymatic Formation of 6,7-Dimethyl-8-ribityllumazine**

**FIG. 4.** $^{13}$C NMR spectrum of riboflavin formed *in vitro* from [2,3,5-$^{13}$C$_3$]ribose 5-phosphate. SC, singly coupled; DC, doubly coupled.

![NMR spectrum of riboflavin](image)

**TABLE V**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific activity</th>
<th>Substrate*</th>
<th>Riboflavin</th>
<th>Lumichromeb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/μmol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1370</td>
<td>1100</td>
<td>30</td>
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<tr>
<td>2</td>
<td>1370</td>
<td>970</td>
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</tbody>
</table>

*5-Amino-6-[1'-$^{14}$C]ribitylamino-2,4(1H,3H)-pyrimidinedione. Obtained by photolysis of riboflavin.

**TABLE VI**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific activity</th>
<th>Substrate*</th>
<th>6,7-Dimethyl-8-ribityllumazineb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/μmol</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>1370</td>
<td>1180</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>1370</td>
<td>1290</td>
<td>21</td>
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</table>

*5-Amino-6-[1'-$^{14}$C]ribitylamino-2,4(1H,3H)-pyrimidinedione. Obtained by photolysis of 6,7-dimethyl-8-ribityllumazine.

**DISCUSSION**

The incorporation of $^{14}$C-labeled pentose phosphates into riboflavin by cell extract of *C. polluermondii* follows accurately the pattern observed in the course of our earlier *in vitro* studies with *A. gossypii*. C1, C2, C3, and C5 of the precursor (ribose phosphate or ribose phosphate) are efficiently incorporated into the heterocyclic moiety, whereas C4 of the precursor is eliminated by an intramolecular skeletal rearrangement.

Generation of the lumazine 2 from ribose 5-phosphate involves two distinct processes: (a) rearrangement of a 5-carbon unit and (b) condensation of the rearrangement product with the pyrimidine precursor. The data provide information on each process.

The experiment with [2,3,5-$^{13}$C$_3$]ribose 5-phosphate demonstrates that the rearrangement occurred in 100% of the labeled molecules. The experiments with both $^{13}$C-labeled substrates indicate that the condensation with 1 proceeds with the expected regiochemistry.

Cursory examination of the data from the incubation with the triple-labeled substrate might lead to the conclusion that the condensation is not entirely regiospecific, as seen by significant $^{13}$C-enrichment of C1*. It appears, however, that
the *1* labeling is due to metabolism of the proffered pentose interchanging C-1 and C-2, perhaps via the pentose phosphate shunt. This idea is supported by the observation that the sum of the enrichments of C1* and C2* equals the enrichments of both C3* and C4* and that the amount of 1*-2* coupling is only what is expected on a statistical basis. If the C1* labeling were due to inversion of the 4-carbon unit, C1* would be coupled to C2* to the same extent as C4* to C3* (i.e. 97%), which is clearly not observed. The interchange of pentose C1 and C2 also explains the presence of singly coupled species at C3*. This interchange also occurred approximately 20% of the time with the sample from [1-14C]ribose 5-phosphate, as well as in previous in vivo experiments.

The specific incorporation rates of carbon from pentose precursors observed in the present experiments present an unresolved problem. Since the 4-carbon unit is duplicated in the last biosynthetic step, 13C-labeled riboflavin should have a relative specific activity of 200% of that of the precursor. The observed values (about 120%) were lower than the expected value. Substantial isotopic dilution has also been observed in the experiment with [1-13C]ribose 5-phosphate, but little dilution occurred in the experiment with [2,3,5-13C3] ribose phosphate. The origin of the isotopic dilution is unknown. The dialyzed cell extracts used in this study contained a small amount of unlabeled riboflavin, but it appears likely that some unlabeled riboflavin was also formed de novo during the enzyme incubation from an unknown precursor present in the cell extract. We expect that this problem will be resolved by further work with purified enzymes instead of crude cell extracts.

Other authors have reported on the formation of riboflavin from 4 by partially purified enzymes from E. coli. This reaction proceeded in the absence of a second substrate, and it has been suggested that the ribitol side chain of 4 was the source of the carbon atoms of the xylene ring (19). In our experiments, the isotope from 1'-14C-labeled 4 was not incorporated into the heterocyclic moieties of 2 and 3, whereas it was incorporated into the side chain without significant dilution. Although it is conceivable that different biosynthetic pathways could operate in the yeast C. guilliermondii and the bacterium E. coli, earlier in vivo experiments with Salmonella typhimurium agree with the biosynthetic pattern reported in this paper. Thus, a mutant of S. typhimurium with an absolute requirement for guanosine incorporated label from [1'-14C]guanosine into the ribitol side chain, but not into the isoalloxazine moiety of riboflavin (48). In vivo studies with B. subtilis have indicated the same biosynthetic pattern as in A. gossypii and C. guilliermondii, i.e. formation of C1* from C1 and extrusion of C4 of the carbohydrate precursor (49). We conclude that a pentose phosphate is the actual precursor of the 4-carbon unit both in bacteria and fungi.

Since various carbohydrate phosphates can serve as substrates in the enzyme reaction studied, the structure of the direct riboflavin precursor remains elusive. In particular, we cannot decide whether it is a pentose phosphate or a pentulose phosphate. Apparently, various carbohydrate phosphates are rapidly interconverted by enzymes present in the crude cell extract.

We have recently obtained evidence for the formation of an aliphatic compound preliminarily designated as "X" which is produced by the cell extract of a C. guilliermondii mutant from ribose phosphate (60). Compound X can be converted to 2 in the presence of 4 by cell extract from a different C. guilliermondii mutant. The structure of compound X has not yet been determined. However, it was shown that the compound is inactivated by treatment with phosphatase suggesting the presence of a phosphoric acid moiety. It is not known whether compound X contains 5 or 4 carbon atoms.

On the basis of the data presented here it is clear that the in vitro system reflects the biosynthetic processes observed in vivo, as opposed to an artefactual production of riboflavin. Thus, we may confidently bring together information gained from in vivo and in vitro studies to suggest a hypothetical scheme for the biogenesis of lumazine 2 (Figs. 5 and 6). The 4-carbon unit is depicted (Fig. 5) as being generated via a radical mechanism, initiated by abstraction of a hydrogen atom from C3 of ribose 5-phosphate. This latter step has precedent, e.g. in the ribonucleotide reductase reaction (61). However, at this stage an equivalent cationic mechanism, initiated by abstraction of a hydride from C3, is equally likely. Condensation of the proposed 4-carbon precursor with 4 to give 2 (Fig. 6) follows a plausible mechanistic pathway, based on known chemistry of these compounds. The proposed mechanism is not in conflict with any of the information currently available on riboflavin biosynthesis; however, a number of alternative mechanisms cannot be ruled out.

It should be noted that the unphosphorylated pyrimidine 4 and its 5'-phosphate 1 can both serve as substrates for the
formation of 2. This could imply that the respective enzyme accepts both pyrimidines. However, it is also possible that the phosphate 1 is enzymatically dephosphorylated by the cell extract prior to incorporation into 2 and riboflavin. Recent studies with a lumazine synthase/riboflavin synthase complex (i.e. "heavy riboflavin synthase") from B. subtilis suggest that the committed precursor for the lumazine 2 is the unphosphorylated pyrimidine. The availability of a cell-free system capable of synthesizing riboflavin from 1 and a pentose phosphate opens the way to the mechanistic investigation of the intriguing reactions involved in this biosynthesis and to the characterization of the intermediates and enzymes involved. The demonstration that the cell-free process shows the same features as the biosynthetic pathway in vivo provides assurance that information gleaned from the in vitro system does relate to the normal biosynthesis of riboflavin in the intact organism.

Acknowledgments—We thank Prof. H. Schirmer, Heidelberg, for helpful discussions. We are indebted to the Los Alamos Stable Isotope Resource, supported under National Institutes of Health Grant RR 02231, for 13C-labeled materials, and to the Ohio State University Chemical Instrument Center for mass spectra. We thank Degussa AG for a generous gift of reagents. The secretarial assistance of Astrid Konig and Angelika Kohne is gratefully acknowledged.

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2 G. Neuberger and A. Bacher, manuscript in preparation.


Continued on next page.
Enzymatic Formation of 6,7-Dimethyl-8-ribityllumazine

**SOLUBILIZATION MATERIAL TO**

**Biophysics of Riboflavin. Enzymatic Formation of 6,7-Dimethyl-8-ribityllumazine from Pentose Phosphates**

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**EXPERIMENTAL PROCEDURES**

Chemicals - 1-Methyl-4-ribitylaminopyrazolin-2-(41,5,6,7)-pyrimidine (27), 3-methyl-4-ribitylaminopyrazolin-2-(41,5,6,7)-pyrimidine (27), 5-ribohydroxynicotinamide (5), 5-ribohydroxynicotinamide 5-phosphate (32), 3-methyl-6-ribohydroxynicotinamide 5-phosphate (26), 5,6,7,8-tetrahydro-5-ribitylamino-2,4(1H,3H)-pyrimidinedione (500), and 6,7-dimethyl-8-ribityllumazine (54) were prepared as described. [1-23C]Glucose, [2-23C]Glycerol, and [1-23C]Ribose, were purchased from Biebricher (

Enzyme Substrates - Ribose 5-phosphate and Plorisil was obtained from Sigma. Aqueous Stable Isotope Resoucou, by standard literature procedures (53,54).

**RESULTS**

- Light riboflavin synthesis of *Nostoc satellit* was purified to a specific activity of 1.05 mol/l. From this preparation, 500 g of glucose 5-phosphate was converted to 500 mg of 6,7-dimethyl-8-ribityllumazine.

- Glucose 5-phosphate dehydrogenase (EC 1.1.1.47) was isolated from *Mycobacterium". The enzyme was purified by chromatography on a Dowex 1 column as described above for barium salt of glucose 6-phosphate. The flow rate of 9.6 mg/min. Preparative centrifugation yielded 6.6 g of a stable salt, which was dissolved in 150 ml of 0.2 M Tris HC1 buffer, pH 7.8, and was stored at 0°C for 24 h. The solution was used immediately.

- Enzyme Substrates - 6-Amino-6-ribitylamino-Z.4(lH,3H)-pyrimidinedione (51) was prepared by catalytic hydrogenation of 6-ribohydroxynicotinamide 5-phosphate (500) to 6-ribohydroxynicotinamide 5-phosphate as described above, the solution was used immediately.

- Propagation of isotopically labeled carbonyl glucose phosphates - Radioactive phosphoglycerate from glucose - Reaction mixtures contained 0.25 M Tris hydrochloride pH 7.8, 10 mM MgCl2, 15 mM NADP*, 15 mM ATP, 5.6 mM Na2SO4, 50 mM NaCl, 27 mM sodium pyruvate, 1.3 mM sodium pyruvate, and 1.3 mM glucose 6-phosphate. The combined yield from o-glycerophosphate dehydrogenase (43) is based on values of 8.8 k/mol based on glucose 6-phosphate on the basis of enzymatic assay (46). This corresponds to an isolation yield of 79.1 % based on the amount of glycerol reacted (65.5 mol/total 6-phosphogluconate) as determined by titration and washings with acetone. The purified crude barium salt solution was then washed with H2O and eluted with a gradient of ammonium formate buffer (gradient of 1 of H2O and 240 ml of 100 mM ammonium formate, pH 3). Precipitation of the barium salt with acetone was attempted, but was unsuccessful. The purification on Dowex 180 was repeated and the fractions containing 8-glycerophosphate, as judged by negative paper phosphate assay, were pooled. Barium acetate (25 mmol) was added, and ethanol was subsequently added to a final concentration of 80 % (v/v). The precipitated barium salt was dissolved in 150 ml of 0.2 M Tris HC1 buffer, pH 7.8, and was stored at 0°C for 24 h. The solution was used immediately.

- Glycerol phosphate dehydrogenase was converted into glucose 6-phosphate in two batches. The reaction mixture was incubated at 30°C for 18 h.

**DISCUSSION**

- The mixture of 5.4 mmol of glucose 6-phosphate and 1.3 mol of 6-phosphogluconate was incubated as described above. The fractions containing the product were then added to 50% ethanol, which was filtered off and washed with 0.01 M NaOH. Ethanol was added to the combined filtrate and washings with acetone. The precipitated crude barium salt solution was purifed by chromatography on a Dowex 1 column as described above for barium salt of glucose 6-phosphate.
ribose oxime were collected on a frosted glass disk by suction and washed three times with 70 ml of ethanol yielding 2.8 mg of white solid. The filtrate was concentrated to dryness and yields yielded approximately 2 mg of ribose oxime which was separately concentrated to 5-nitro-6-[(13C)ribitylamino]-4,1,3-benzoxazol-2(3H)-yridindleminedine.

[1-13C]Ribitylamine - A suspension of 1.1 mg of PDC in 2 ml of acetic acid was hydrolyzed for 20 min. [1-13C]Ribose oxime (2.6 mg) was added and hydrolyzed for 12 h at room temperature and atmospheric pressure. Sodium acetate was removed by evaporation, and the solution was evaporated to dryness under reduced pressure. The oily residue was dissolved in 10 ml of water and placed on a column of AG 50 W-X 8 (10 mm, 100-200 mesh). The column was washed with 100 ml of deionized water. Ribitylamine was subsequently eluted with 50 ml of 0.1 M HCl. The eluent was evaporated to dryness under reduced pressure. Deionized water was added and evaporation was repeated three times.

5-nitro-6-[(13C)ribitylamino]-2,4(1H,3H)-pyridindinedione - A solution containing 1.3 mg of 5-nitro-6-[(13C)ribitylamino]-2,4(1H,3H)-pyridinedione in 350 ml of water containing 0.1 M sodium acetate was added. Ribitylamine (2.0 mmol dissolved in 10 ml of 0.1 M, 1.5 ml) was added and the solution was kept at 40 °C for 24 h. The solvent was evaporated under reduced pressure, and the residue was dissolved in 25 ml of deionized water. The solution was passed through a preparative HPLC column (Lichrosorb RP18, 15 x 250 mm; eluent: 15% methanol, formamide pH 5.0; flow rate: 10-12 ml/min; fractions were collected and lyophilized. Yield: 1.10 mmol. Specific activity: 1320 dpm/nmol. Radiochemical yield based on [1-13C]Ribose, 13.2%.

The second crop of [1-13C]Ribose oxime obtained at the end of step 1 was separately carried through the subsequent steps yielding an additional amount of 8 mmol of 5-nitro-6-[(13C)ribitylamino]-2,4(1H,3H)-pyridinidinedione with a specific activity of 1370 dpm/nmol. The total radiochemical yield based on ribose was 15%. Both batches appeared pure as judged by analytical HPLC and thin layer chromatography (reolidol, 1-butanol/acetic acid/water 50:45:5, v/v; Rf = 0.62). They showed the expected absorption spectrum λmax = 227 nm, 352 nm, 1727/693 (1% at 1%).

Enzyme studies

All experiments were performed in dim light to minimize photolysis of products. All enzymatic reactions were freshly prepared for each experiment, and efforts were made to minimize exposure to molecular oxygen.

Riboflavin from [1-13C]ribosamine 5-phosphate - Assay mixtures contained 0.1 M Tris hydrochloride pH 7.5, 15 ml sodium acetate, 1 ml dithiothreitol, 1 ml 0.1 M sodium phosphate, 1 ml ribosomal ribose oxime, 1000 U of light riboflavin synthase from M. smegmatis, and dialyzed cell extract from C. guilliermondii ATCC 29585 (2 mg of protein) in a total volume of 2 ml. The mixture was incubated for 1 h at 30 °C. An aliquot (100 ml) was retrieved for bioassay of riboflavin using a 0.5 ml 1% yeast solution diluted 1:100 in water. A 1 ml sample of deionized water was added as a carrier to the assay mixture, and the solution was passed through a column of Florisil (16.5 x 1 cm). The column was washed with 15 ml of deionized water. Fluorometric material was eluted with 5 ml of acetone/2 M NaOH (1:1, v/v). The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in 0.1 ml of deionized water and filtered through a 0.22 μm filter. The filtrate was used for fluorescence detection with a 0.1 ml sample of aqueous formamide (7% of formic acid) in 0.1 ml formamide formate buffer pH 3.8. The effluent was monitored photometrically (254 and 405 nm). The fraction containing 6,7-dimethyl-8-ribitylflavinamine (transient time, 32 min) was collected and lyophilized.

Degradation

Photochemical degradation of riboflavin - Riboflavin was dissolved in buffer containing 0.1 M formic acid and 0.25 M aqueous formate pH 3.8. The solution was illuminated for 2 h by a 300 W lamp (distance, 0.1 m). The solution was passed through a SPFC column (Hercules 10 C4, 0.4 x 25 cm, flow rate 2 ml/min) to elute 0.1 M aqueous formate buffer pH 3.8. Absorbance was monitored at 254 and 365 nm. The fraction containing 6,7-dimethyl-8-ribitylflavinamine (transient time, 17 min) was collected and lyophilized.

Photochemical degradation of 6,7-dimethyl-8-ribitylflavinamine - A solution of 6,7-dimethyl-8-ribitylflavinamine (0.1 M aqueous formate buffer pH 3.8) was illuminated for 1 h as described above. The solution was passed through a SPFC column (Hercules 10 C4, 0.4 x 25 cm, flow rate, 1 ml/min) to elute 0.1 M aqueous formate buffer pH 3.8. Absorbance was monitored at 254 and 365 nm. The fraction containing 6,7-dimethyl-8-ribitylflavinamine (transient time, 25 min) was collected and lyophilized.

NMR Spectroscopy

4-decoupled 1H/13C-NMR spectra were obtained at 7.0 T on a Bruker WH-300 MRI spectrometer. Samples of riboflavin were dissolved in approximately 0.6 ml of deuterio-D2O (0.0-20% D2O). The spectral acquisition parameters were: pulse angle = 60°, repetition time = 2 sec, spectral width = 15,15 KHz 32 K data sets, 1.0 Hz line broadening. Chemical shifts are referenced to tetramethylsilane.

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