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Biosynthesis of Riboflavin: Mechanism of Formation of the Ribitylamino Linkage[†]

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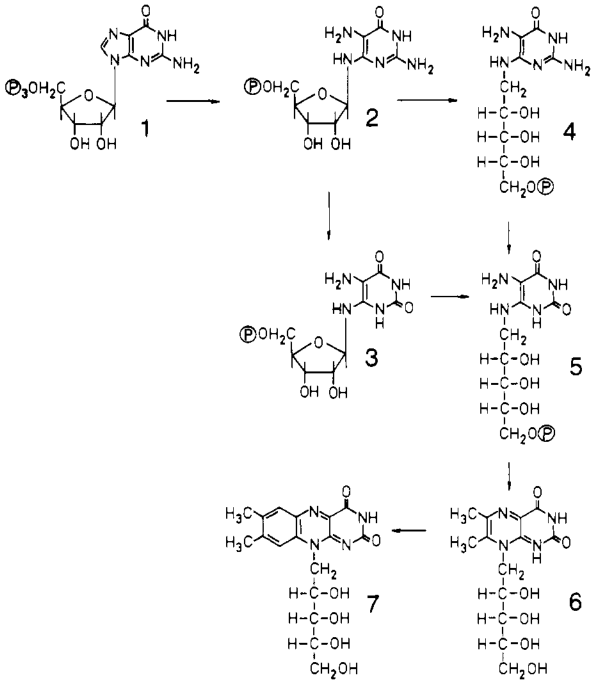
ABSTRACT: Feeding experiments with *Ashbya gossypii* followed by NMR analysis of the resulting riboflavin showed incorporation of deuterium from D-[2-^{2}H]ribose at C-2′ and from D-[1-^{2}H]ribose in the *pro-R* position at C-1′ of the ribityl side chain. The results rule out an Amadori rearrangement mechanism for the reduction of the ribosylamino to the ribitylamino linkage and point to formation of a Schiff base that is reduced stereospecifically opposite to the face from which the oxygen has departed. As prerequisite for the analysis, the ^{1}H NMR signals for the *pro-R* and *pro-S* hydrogens at C-1′ of 6,7-dimethyl-8-ribityllumazine and riboflavin and its tetraacetate were assigned with the aid of synthetic stereospecifically deuteriated samples.

The biosynthesis of riboflavin (**7**) from GTP (**1**) (Scheme I) is initiated by the enzyme GTP cyclohydrolase II, which excises C-8 of the imidazole ring and removes two of the phosphate groups of GTP to generate 2,5-diamino-6-(ribosylamino)-4(3*H*)-pyrimidinone 5′-phosphate (**2**) [for reviews, see Brown and Williamson (1982) and Le Van et al. (1985)]. Conversion of **2** into 5-amino-6-(ribitylamino)-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate (**5**) involves two steps, deamination at C-2 and reduction of the ribosylamino to the ribitylamino linkage, which take place in different orders in bacteria and fungi. In *Escherichia coli*, the pyrimidine ring is first deaminated to give **3**, which is then reduced to **5** with NADPH as the reducing agent (Burrows & Brown, 1979). In yeast, reduction of the ribosylamino linkage first produces **4**, which is then deaminated to **5** (Oltmanns & Bacher, 1972; Nielsen & Bacher, 1981). The reducing agent utilized in yeast has not been determined. The further conversion of **5** into **7** has been the subject of considerable investigation over the years [for reviews, see Plaut et al. (1974), Brown and Williamson (1982), and Bacher et al. (1983)]. Recent results indicate that it involves condensation of the dephospho derivative of **5** with a phosphate ester, compound X (Neuberger & Bacher, 1985, 1986), which is derived from a pentose/pentulose phosphate by an unusual rearrangement (Bacher et al., 1985; Nielsen et al., 1986).

Scheme I: Biosynthesis of Riboflavin



In this paper we report on an aspect of one of the early steps in riboflavin biosynthesis, the mechanism and stereochemistry of the reduction of the ribosylamino to the ribitylamino linkage.

Materials and Methods

General Methods. Feeding experiments with *Ashbya gossypii* were carried out as previously described (Bacher et al., 1983). Briefly, labeled ribose (1.2 g of [1-^{2}H]ribose or 1.31 g of [2-^{2}H]ribose) was added to 0.22 L of a culture of *A. gossypii* and incubated with shaking for 24 h. Riboflavin was isolated and converted to the tetraacetate for NMR spectroscopy. ^{1}H NMR spectra and ^{1}H-decoupled ^{13}C and ^{2}H NMR spectra were recorded on a Bruker WM-300 spectrometer at 7.0 T. Chemical shifts are referenced to tetramethylsilane. For the synthesis of D-[1-^{2}H]- and D-[2-^{2}H]-ribose, the cyanohydrin from D-erythrose was reduced with ^{2}H gas in $^{2}H_2O$ (Serianni et al., 1982a), the resulting D-[1-^{2}H]ribose and D-[1-^{2}H]arabinose were separated by ion-exchange chromatography, and the latter was isomerized to D-[2-^{2}H]ribose as described (Hayes et al., 1982; Serianni et al., 1982b).

Synthesis of (1′R)-[1′-^{2}H]Riboflavin. D-Ribose (5.12 g, 34 mmol) was dissolved in 100 mL of methanol, (*S*)-α-phenylethylamine (4.13 g, 34 mmol) was then added, the reaction flask was capped tightly and the mixture stirred for 3 h, and then $NaBD_3CN$ (2.5 g, 37 mmol) was added to the mixture, which was stirred overnight at room temperature. Acetic acid (2 mL) was added to give a pH of about 7, and after being allowed to stand for 8 h, the mixture was passed through a column of Dowex 50W-X8 (H^+) in methanol. The column was washed with methanol and the product eluted with

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15% concentrated NH_4OH in methanol. Final purification was done by preparative-layer chromatography on silica gel with MeOH–EtOH (1:3) containing 1.8% concentrated NH_4OH [R_f 0.32; yield 6.61 g (76%)].

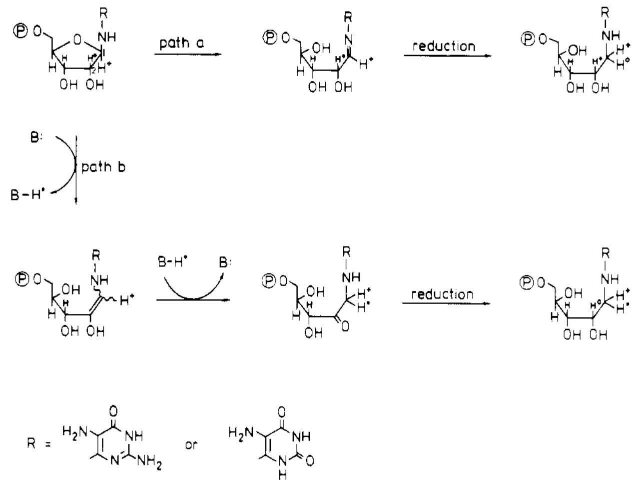
1-Deoxy-1-[(*S*)-(α-phenylethyl)amino]-D-[1-$^{2}H_{1}$]ribose (1.2 g) was dissolved in 15 mL of ethanol and 2 mL of concentrated HCl, 1 g of 10% Pd–C was added, and the mixture was hydrogenated in a Parr apparatus at 45 psi for 1 week. The catalyst was filtered off, the filtrate evaporated, and the residue taken up in 10 mL of H_2O. Ethylbenzene was removed by extraction 2× with 20 mL of ether and 2× with 20 mL of benzene, and the aqueous solution was evaporated to give 0.55 g of a syrup of [1-$^{2}H_{1}$]ribitylamine. Final purification was by chromatography on a column of silica gel with 10% concentrated NH_4OH in MeOH as eluent. ^{1}H NMR showed a ratio of the peaks at 2.69 ppm (H-1*S*) and 2.88 ppm (H-1*R*) of about 75:25. The product from the analogous reaction with (*R*)-α-phenylethylamine contained an almost equal ratio of the 1*R* and 1*S* epimers.

The (1*R*)-[1-$^{2}H_{1}$]ribitylamine (200 mg, 1.3 mmol) was dissolved in 4 mL of water with 192 mg (1 mmol) of 5-nitro-6-chloro-2,4(1*H*,3*H*)-pyrimidinedione in 4 mL of ethanol (Cresswell & Wood, 1960), and the pH was adjusted to 8.0. The solution was left at room temperature for 2 days and placed on a column of Dowex 1-X8 (formate form, 1 × 15 cm). The column was developed with 0.1 M formic acid. Fractions were pooled and concentrated under reduced pressure. The residue was dissolved in 10 mL of water and hydrogenated over palladized charcoal at room temperature and atmospheric pressure. Diacetyl (1 mL, 11.4 mmol) was added, and the mixture was kept at room temperature for 1 h. (1′*R*)-[1′-$^{2}H_{1}$]-6,7-Dimethyl-8-ribityllumazine (**6**) (57 mg, 0.175 mmol) was purified as described elsewhere (Bacher, 1986).

Compound **6** (25 mg, 0.077 mmol) was dissolved in 40 mL of 0.1 M phosphate buffer, pH 7, containing 10 mM sodium sulfite, 10 mM ethylenediaminetetraacetic acid (EDTA), and 76 000 units of light riboflavin synthase from *Bacillus subtilis* (sp act. 100 000 units/mg), and 5 mM diacetyl was added. The solution was incubated at 37 °C for 2 h and passed through a column of Florisil (2 × 8 cm). The column was washed with 200 mL of water. The yellow fluorescent material was eluted with acetone/2 M NH_4OH (1:1 v/v). The solution was evaporated to dryness under reduced pressure. The residue was dissolved in 5 mL of H_2O and placed on a column of Dowex W-X8 (H^+ form, 1 × 18 cm), which was developed with H_2O. Fractions were pooled and concentrated to dryness under reduced pressure. The residue was crystallized from 3 mL of H_2O, yielding 20 mg (0.053 mmol) of (1′*R*)-[1′-$^{2}H_{1}$]-**7**, which was acetylated to the tetraacetate. ^{1}H NMR: (1′*R*)-[1′-$^{2}H_{1}$]-**6** ([$^{2}H_{6}$]DMSO) 4.41 ppm (H-1′*S*, 77%) and 4.69 ppm (H-1′*R*, 23%). ^{1}H NMR: (1′*R*)-[1′-$^{2}H_{1}$]-**7** ([$^{2}H_{6}$]DMSO) 4.58 ppm (H-1′*R*, 24.5%) and 4.79 ppm (H-1′*S*, 75.5%). ^{1}H NMR: (1′*R*)-[1′-$^{2}H_{1}$]-**7** tetraacetate ($CDBr_3$ at 120 °C) 4.9 ppm (H-1′*R*, 24%) and 5.2 ppm (H-1′*S*, 76%) ^{2}H NMR: (1′*R*)-[1′-$^{2}H_{1}$]-**7** tetraacetate ($CHBr_3$ at 120 °C) 4.9 ppm (H-1′*R*, 80%) and 5.2 ppm (H-1′*S*, 20%).

Degradation of (1R)-[1-$^{2}H_{1}$]Ribitylamine. (1*R*)-[1-$^{2}H_{1}$]-Ribitylamine (0.28 g) and (−)-(1*S*,4*R*)-ω-camphanoyl chloride (0.47 g, 1.2 equiv) were each dissolved in 10 mL of dry dimethylformamide. The latter was added dropwise to the solution of ribitylamine, followed by 1 mL of triethylamine. The solution was then stirred overnight at 50 °C, water (10 mL) was added to destroy any remaining camphanoyl chloride, and the solution was concentrated on a rotary evaporator under an oil pump vacuum. The residue was dissolved in 20 mL of methanol, passed successively through columns (2 × 10 cm) of Dowex 50W-X8 and Dowex 1-X8 in methanol, and concentrated again. After further purification on a silica gel column [eluent: MeOH–$CHCl_3$ (2:5)], the product *N*-ribitylcamphanamide was obtained as a colorless syrup, which crystallized after a few days: yield 0.19 g (32%); mp 63~67 °C.

Scheme II: Two Possible Mechanisms for Reduction of the Ribosylamino to the Ribitylamino Moiety in Riboflavin Biosynthesis[a]



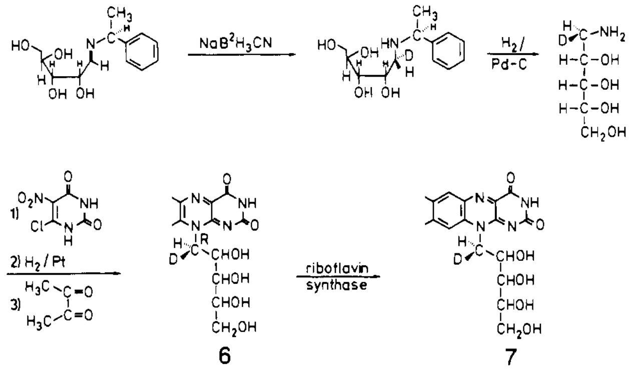
[a] H° designates a hydrogen atom introduced by reduction.

N-Ribityl-(−)-camphanamide (70 mg) was dissolved in 30 mL of H_2O; 10 mL each of 0.2 M sodium acetate and 0.2 M acetic acid was added, followed by 30 mL of 0.1 N $NaIO_4$ and water to 100 mL. The mixture (pH 4.5) was stirred 2 h in the dark at a temperature between 5 and 15 °C. Excess $NaIO_4$ was destroyed by the addition of ethylene glycol, and the solution was passed through a column of XAD-4 (3.5 × 18 cm), which was washed with 1 L of water. The desired product was eluted with 85% aqueous methanol and passed through a column of AG 1-X8 (2 × 15 cm). The effluent was evaporated to a syrup, 3 mL of bromine and 10 mL of ethyl acetate were added, and the mixture stirred overnight. Excess bromine was removed with a stream of nitrogen, $AgNO_3$ was added, and the precipitate of AgBr was removed by filtration. Diazomethane in ether (0.1 N) was added until a yellow color persisted (2 mL), and excess CH_2N_2 was removed in a stream of nitrogen. Gas chromatography–mass spectrum (GC–MS) analysis showed the presence of several components, one of which matched authentic camphanoylglycine methyl ester (Armarego et al., 1976) in retention time and fragmentation pattern. The product was purified by preparative-layer chromatography [silica gel, ether–petroleum ether (1:1), R_f 0.43). ^{1}H NMR ($CDCl_3$): 3.95 ppm (H-2*R*, 33%) and 4.15 ppm (H-2*S*, 67%).

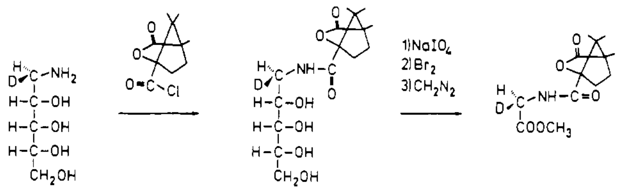
RESULTS AND DISCUSSION

Two plausible mechanisms can be formulated for the reduction of the ribosylamino to the ribitylamino function (Scheme II). One path (a) involves opening of the furanose ring by abstraction of a proton from nitrogen followed by direct reduction of the resulting Schiff base. Alternatively (path b), the process may involve an Amadori rearrangement (Paulsen & Pflughaupt, 1980), initiated by ring opening with abstraction of a proton from C-2, followed by reduction of the C-2 carbonyl group of the resulting ribulosylamine. The dephospho analogue of the expected Amadori rearrangement product, 5-amino-6-[(1-deoxyribulosyl)amino]-2,4(1*H*,3*H*)-pyrimidinedione, has been reported as one of the products

Scheme III: Synthesis of Authentic (1′*R*)-[1′-$^2H_1$]-**6** and -**7** for ^1H NMR Assignment of 1′-Hydrogens



Scheme IV: Degradation of (1′*R*)-[1′-$^2H_1$]Ribitylamine



accumulated by mutants of *B. subtilis* (Bresler et al., 1977), leading to the suggestion that the reaction proceeds by path b. As illustrated in Scheme II, the two mechanisms differ in the fate and origin of various hydrogens in the system. The hydride delivered by a reduced pyridine nucleotide would appear either at C-1 (path a) or C-2 (path b) of the product. The hydrogen at C-2 of the substrate would on path a be retained at that position, whereas on path b it would be either eliminated or transferred, partially or totally, to C-1 if the Amadori rearrangement is catalyzed by a single base on the enzyme with proton recycling. The hydrogen from C-1 of the substrate would, in either case, be retained at C-1 but may occupy either the *pro-R* or the *pro-S* position in the product. Amadori rearrangement by a single base mechanism would suggest proton abstraction and migration opposite to the face from which the oxygen has departed, placing the original H-1 of the substrate in the *pro-R* position in the product, whereas path a allows no prediction regarding stereochemistry.

We decided to attempt to distinguish between the two mechanisms by feeding D-[1-^2H]- and D-[2-^2H]ribose to cultures of the flavinogenic fungus *A. gossypii* and determining the incorporation of deuterium into the ribityl side chain of **7**. The two deuteriated ribose species were synthesized according to the procedures worked out by Barker and colleagues (Hayes et al., 1982; Serianni et al., 1982a). A Kiliani–Fischer synthesis starting from D-erythrose, using deuterium gas in ^2H_2O for the reduction of the cyanohydrin, gave a mixture of D-[1-^2H]ribose and D-[1-^2H]arabinose, which were readily separated by ion-exchange chromatography. Treatment of the arabinose obtained with molybdate produced an equilibrium mixture of D-[1-^2H]arabinose and D-[2-^2H]ribose, which were again separated. In this way we obtained 1.14 g of D-[1-^2H]ribose and 1.28 g of D-[2-^2H]ribose, both as syrups. ^1H and ^{13}C NMR confirmed that they were >90% deuteriated in the expected positions.

A prerequisite for the analysis of the samples of **7** from the projected feeding experiments is the availability of unequivocal assignments for the relevant signals in the ^1H NMR spectrum of **7** and/or its tetraacetate. A partial ^1H NMR analysis of the ribityl side chain of **7** in ^2H_2O has been reported (Williamson & Edmondson, 1986), including an assignment for H-2′, but no assignments for H-1′*R* and H-1′*S* were available. We have confirmed the published assignments and extended them to the tetraacetate of **7** (P. J. Keller, D. H. Brown, and A. Bacher, unpublished work). To assign the signals for the two diastereotopic hydrogens at C-1′ of these compounds, we synthesized a sample of **7** deuteriated stereoselectively at C-1′, as shown in Scheme III. Asymmetric reductive amination of D-ribose with (*S*)-α-phenylethylamine and sodium cyanoboro[^2H]hydride followed by hydrogenolysis gave [1-$^2H_1$]ribitylamine containing about 75% of one and 25% of the other C-1′ epimer, as determined by ^1H NMR. The corresponding reaction wih the (*R*)-amine gave almost equal amounts of the two C-1 epimers. Evidently, in the case of the (*S*)-amine, the asymmetric inductions exerted by the amine component and by the sugar reinforce each other, whereas with the (*R*)-amine they counteract each other. On the basis of literature analogy (Nichols et al., 1973), one would expect the ribitylamine generated by NaB^2H_3CN reduction of the Schiff base with (*S*)-α-phenylethylamine to have predominantly 1*R* configuration; however, the presence of other chiral centers in the Schiff base makes this prediction somewhat tenuous. The configuration of the ribitylamine was therefore confirmed by conversion of an aliquot to the (−)-camphanamide, which was degraded to (−)-camphanoyl[2-$^2H_1$]glycine methyl ester (Scheme IV). ^1H NMR comparison of the latter with the published data (Armarego et al., 1976) on the authentic derivatives of (*R*)- and (*S*)-[2-$^2H_1$]glycine unequivocally established the predominant configuration at C-1 of the ribitylamine as *R*. The bulk of the (1*R*)-[1-$^2H_1$]ribitylamine was then used to synthesize riboflavin tetraacetate via (1′*R*)-[1′-$^2H_1$]-**6** and (1′*R*)-[1′-$^2H_1$]-**7**. The ^1H NMR spectra of the latter three compounds were recorded, establishing the following assignments (**6** and **7** in DMSO; **7** tetraacetate in $CDBr_3$): (*pro*-1′*R*/*pro*-1′*S* hydrogen) **6**, 4.69/4.41 ppm; **7**, 4.58/4.79 ppm; **7** tetraacetate, 4.9/5.2 ppm. It is noteworthy that the order of the two signals changes in going from **6** to **7** but is the same in **7** and its tetraacetate.

With these assignments at hand, the two deuteriated ribose samples were fed to cultures of *A. gossypii*. [1-^2H]Ribose (1.2 g to 220 mL) gave 9.5 mg of **7**, and [2-^2H]ribose (1.31 g to 220 mL) gave 58.7 mg of **7**; both samples were converted to the tetraacetate for NMR analysis. The ^1H NMR spectrum of the material from [2-$^2H_1$]ribose showed a signal at 5.64 ppm (H-2′), which integrated for slightly less than one proton, and the ^2H NMR spectrum showed a very broad peak at 5.64 ppm. Most informative, however, was the ^1H and ^2H broad-band decoupled ^{13}C NMR spectrum. Although the ^2H-shifted signal for C-2′ was obscured by the C-4′ signal, the signal for C-3′ showed an isotope satellite (12% area) due to a 4.2-Hz deuterium β-shift (Abell & Staunton, 1981) (Figure 1). A corresponding satellite was observed for the C-1′ signal, confirming the presence of 12% deuterium at C-2′ of this sample of riboflavin.

The sample of **7** tetraacetate from [1-^2H]ribose showed in the ^1H NMR spectrum a ratio of the signals at 4.9 and 5.2 ppm of 0.89:1 and in the ^2H NMR spectrum a pronounced signal at 4.9 ppm (Figure 2), revealing the presence of about 10–12% deuterium at C-1′, which occupies the *pro-R* position. The deuterium distribution in the two samples of **7** is shown in Scheme V.

On the basis of the arguments put forth above, the results rule out an Amadori rearrangement mechanism (path b) for the reduction of the ribosylamino linkage, because such a mechanism would result in either the complete loss of the deuterium from C-2 of ribose or its transfer to C-1. It is incompatible with the observed retention of deuterium at C-2′

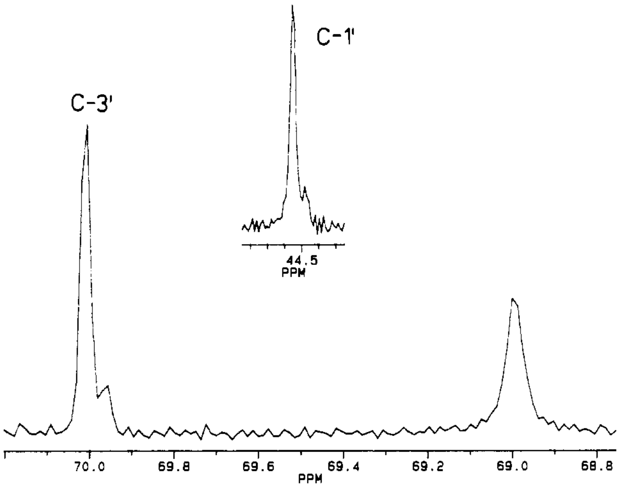


FIGURE 1: Section of the ^1H broad-band-decoupled ^{13}C NMR spectrum of **7** tetraacetate obtained biosynthetically from D-[2-^2H]ribose.

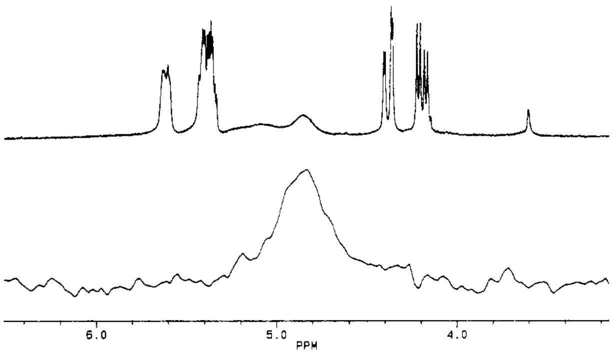
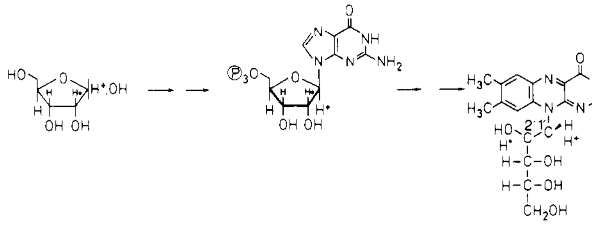


FIGURE 2: Section of ^1H NMR spectrum of unlabeled **7** tetraacetate (top) and ^2H NMR spectrum of **7** tetraacetate obtained biosynthetically from D-[1-^2H]ribose (bottom).

Scheme V: Stereochemistry of Formation of the Ribitylamino Linkage of Riboflavin from Ribose via Guanosine Triphosphate



of **7**. As an alternative, the results point to path a (Scheme II), the direct reduction of a Schiff base generated by opening of the furanose ring. The observed stereochemistry, replacement of the oxygen by hydrogen with inversion, indicates that the Schiff base is reduced on the face opposite the one on which the oxygen has departed. No mechanistic conclusions can be drawn from this observed stereochemistry; rather, it probably reflects a steric preference which minimizes crowding of functional groups in the enzyme active site. The mechanism and stereochemistry deduced in these experiments relate to the fungal pathway of **7** formation; whether the bacterial route, in which the order of reduction and deamination is reversed, operates in the same manner remains to be established.

Registry No. Riboflavin, 83-88-5; ribose, 50-69-1.

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