Studies on the Mechanism of Elimination of Protons from the Methyl Groups of 6,7-Dimethyl-8-ribityllumazine by Riboflavin Synthetase*

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ABSTRACT: Studies with 6,7-dimethyl-8-(1′-D-ribityl)lumazine and certain analogs selectively labeled with hydrogen isotopes in the methyl groups support a mechanism of riboflavin synthetase involving elimination of protons at the 7-methyl group and carbon–carbon bond formation between it and carbon 7 of a second molecule of the lumazine, with subsequent ring closure between the 6-methyl group and carbon 6 to form the *o*-xylene ring of riboflavin. The rates of enzymic conversion of 6-methyl-7-deuteriomethyl-8-ribityllumazine and 6-deuteriomethyl-7-methyl-8-ribityllumazine into riboflavin are 80 and 20% of that of the unlabeled substrate, respectively. While V_{max} of 6-deuteriomethyl-7-methyl-8-ribityllumazine is lower than that of the unlabeled substrate, the values of k_m are nearly identical. The 6-deuteriomethyl derivative is a competitive inhibitor of the enzymic conversion of the unlabeled substrate, yielding about equal values of k_i and k_m. Inhibition is not due to product inhibition, since inhibition by riboflavin formed from labeled and unlabeled substrates is essentially the same. With tritiated lumazines, in which the predominant labeled species is likely to contain a single atom of tritium per selectively labeled methyl group, preferential elimination of protons over tritions into water and retention of the label in riboflavin occurs with 6-tritiomethyl-7-methyl-8-ribityllumazine whereas there is relatively little isotope discrimination with 6-methyl-7-tritiomethyl-8-ribityllumazine. The loss of protons occurs from the methyl groups of the lumazine molecule which accepts the 4-carbon moiety, since the methyl groups of riboflavin formed retain essentially all of the tritium which should have been transferred from the labeled donor molecule of lumazine. No substantial enzymic enhancement of trition elimination from 6-methyl-7-tritiomethyl-8-ribityllumazine occurs at low pH where riboflavin formation is absent or minimal; at neutrality (pH 6.8), where riboflavin production is maximal, the rates of incorporation of tritium into water and flavin formation are about equal; at pH 8.8 the loss of tritions is 10–15 times faster than riboflavin formation. At pH 6.8 with substrates such as 6-methyl-7-tritiomethyl-8-[1′-(5′-deoxy-D-ribityl)]lumazine and 6-deuteriomethyl-7-tritiomethyl-8-ribityllumazine, where k_m is about the same as that of 6,7-dimethyl-8-ribityllumazine but V_{max} is markedly less, the velocities of tritium–hydrogen exchange are faster than flavin formation, although the apparent first-order rate constants for tritium–hydrogen exchange are about the same for 6-methyl-7-tritiomethyl-8-ribityllumazine and the analogs. 6-Methyl-7-tritiomethyl-8-(1′-D-xylityl)lumazine which is not converted into flavin but is a competitive inhibitor and forms complexes with the enzyme, participates in tritium–hydrogen exchange. The relationship between substrate specificity and the exchange reaction is shown by the observation that the enzyme does not increase liberation of tritions into water from 6-methyl-7-tritiomethyl-8-(1′-L-xylityl)lumazine which does not interact with the enzyme. Furthermore, proportional inhibition of riboflavin synthesis and trition elimination from 6-methyl-7-tritiomethyl-8-ribityllumazine has been obtained with certain sulfhydryl binding reagents (*p*-mercuribenzenesulfonate, 5,5′-dithiobis(2-nitrobenzoate), and iodoacetamide) further indicating that the exchange reaction and flavin formation are catalyzed by the same protein. A mechanism of riboflavin synthetase has been proposed in which elimination of protons from the 7-methyl group reflects reversible steps occurring at the enzyme site binding that molecule of 6,7-dimethyl-8-ribityllumazine which accepts the 4-carbon moiety from a second molecule of the lumazine also bound to the enzyme. Intermolecular carbon–carbon bond formation between the 7-methyl group and carbon 7 of two molecules of 6,7-dimethyl-8-ribityllumazine probably precedes ring closure between the corresponding 6-methyl group and carbon 6. The latter step(s) appears to be rate limiting in riboflavin formation. An improved method for the purification of riboflavin synthetase from yeast is reported.

Studies on the enzymic synthesis of riboflavin from 6,7-dimethyl-8-(1′-D-ribityl)lumazine[1] have shown that four carbon atoms (carbon atoms 6 and 7 and the neighboring methyl group) are transferred from one molecule of the lumazine to a second molecule of the lumazine to form the *o*-xylene portion of riboflavin (Plaut, 1963; Wacker *et al.*, 1964). The mechanism of this reaction must involve the loss of two protons from each of the two methyl groups of that molecule of 6,7-dimethyl-8-ribityllumazine which acts as the

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[1] Throughout the text the less cumbersome name for this compound, 6,7-dimethyl-8-ribityllumazine, will be used.

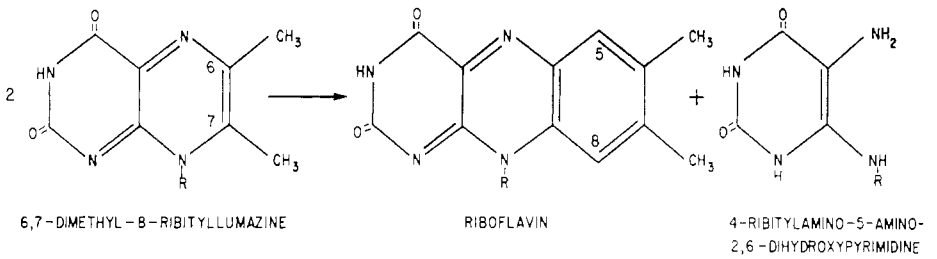

FIGURE 1: Stoichiometry of the riboflavin synthetase reaction; R = ribityl group.

acceptor of the 4-carbon moiety (see Figure 1); however, transfer of the 4-carbon fragment need involve no such obligatory loss of protons.

Riboflavin synthetase has been purified several thousand-fold from yeast extract (Harvey and Plaut, 1966). A method is reported here leading to a final preparation of somewhat higher specific activity in a greatly improved yield. With the purified riboflavin synthetase from yeast and that from other microorganisms (Plaut, 1963), it still appears that only a single enzyme protein is required for the conversion of 6,7-dimethyl-8-ribityllumazine into riboflavin. No intermediate of the reaction has been detected (Harvey and Plaut, 1966). Further studies of the mechanism of the reaction showed that a specific nonenzymic exchange of hydrogen and deuterium between water and the 7-methyl group, but not the 6-methyl group, of 6,7-dimethyl-8-ribityllumazine occurs (Beach and Plaut, 1969).

The nonenzymic exchange made possible the synthesis of 6,7-dimethyl-8-ribityllumazine carrying various isotopes of hydrogen in specific methyl groups. These isotopically labeled compounds were useful in elucidation of certain steps of the riboflavin synthetase reaction. Evidence presented here shows that the loss of protons from the 7-methyl group and the exchange reaction is catalyzed by purified riboflavin synthetase. Elimination of protons from the 7-methyl group probably precedes that from the 6-methyl group, the latter step appears to be rate limiting in the enzymic formation of riboflavin.

Experimental Procedures

Materials. 6,7-Dimethyl-8-(1′-D-ribityl)lumazine, 6,7-dimethyl-8-(1′-D-xylityl)lumazine, 6,7-dimethyl-8-(1′-L-xylityl)-lumazine, and 6,7-dimethyl-8-[1′-(5′-deoxy-D-ribityl)]lumazine were prepared as described previously (Winestock and Plaut, 1961). Lumazine derivatives labeled with tritium in the methyl groups at positions 6 or 7 were prepared as were the deuterium-containing substances (Beach and Plaut, 1970).

The following materials were purchased from the suppliers indicated: Celite 560 (Johns Manville), Darco G60 (Pfanstiehl Lab. Inc.), Lloyd's reagent (Hartman–Leddon Co.), Whatman cellulose powder Cf11 (Reeves Angel), TEAE-cellulose (Bio-Rad), Red Star active dry yeast (Universal Foods, Milwaukee, Wis.), tritium oxide and deuterium oxide (New England Nuclear), dithioerythritol and *p*-mercuribenzenesulfonate (Sigma), riboflavin (Merck), enzyme grade ammonium sulfate (Mann), and mercaptoethanol (Eastman, White Label). All other chemicals used were of reagent grade.

Deionized water redistilled from glass was used in preparing the enzyme and in assays.

Methods

Incorporation of Radioactivity from Tritiated Lumazine Derivatives into Water and Flavins. INCUBATION. Nonenzymic. The tritiated substance is weighed on a microbalance and dissolved in the appropriate buffer (equilibrated at 25°) to a final concentration of approximately 50 μg of substance/ml of reaction mixture. A zero time sample (0.20 ml) is applied to the charcoal–Celite column immediately after dissolving the compound. The solution is incubated at 25° and 0.20-ml aliquots are applied to charcoal–Celite columns at intervals. The columns are developed as described below immediately after application of sample; the tritium content of the water effluent is determined in a scintillation counter.

Concentration of the lumazine derivative is determined spectrophotometrically at 407 mμ (ϵ 10,300).

Enzymic. The lumazine derivatives are dissolved in buffer and a zero time sample is placed on a charcoal–Celite column as described for the procedure in the absence of enzyme. A 3.00-ml sample of the lumazine solution is then added to a cuvet containing enzyme (usually 0.015 ml of enzyme/ml of reaction mixture producing up to 50 nmoles of riboflavin/ml per hr) equilibrated to 25° in the thermostated cuvet holder of the spectrophotometer. The time elapsed between dissolving the lumazine in buffer and the start of the reaction by addition of enzyme is about 1 min. (This time lapse has been measured in each experiment by timers activated by foot switches. When necessary the contribution of the nonenzymic tritium exchange during this initial time interval could be estimated.)

Aliquots of 0.20 ml are removed and placed on columns of charcoal–Celite at appropriate intervals for the determination of incorporation of tritium into water.

Formation of riboflavin is measured at 470 mμ and recorded continuously at a chart speed of 2 in./min throughout the incubation period.

The reaction is terminated by the addition of 15% trichloroacetic acid to a final concentration of 5%. Coagulated protein is removed by centrifugation, the flavin content of the clear supernatant fluid is determined spectrophotometrically at 470 mμ (ϵ 10,300), and flavin is purified by column chromatography on Lloyd's reagent (Plaut, 1963) and thin-layer chromatography as described below.

Determination of Incorporation of Tritium into Water and Flavin. INCORPORATION INTO WATER. A suspension is prepared containing 1.25 g of charcoal (Darco G60) and 2.5 g of

Celite 560 in 100 ml of 0.05 M imidazole-HCl buffer at pH 6.8. A disposable pipet (0.5 cm i.d.) is plugged with glass wool and a 0.5 × 1.5 cm column of charcoal–Celite is formed from about 4 ml of the suspension of adsorbents. The column is washed with 8 ml of the buffer before use.

A 0.20-ml aliquot of reaction mixture is placed on the column. Two 2.00-ml portions of 0.05 M imidazole-HCl at pH 6.8 are then passed through the column with air pressure (flow rate of 2.0 ml/30 sec); the effluents are collected separately and analyzed for radioactivity.

Fluorometric measurements show that when 10 μg of 6,7-dimethyl-8-ribityllumazine was applied to such columns, less than 0.001% of the lumazine applied was recovered in effluents. Radioactivity from buffers containing T_2O was recovered completely.

Separation of Riboflavin from Other Components of the Reaction Mixture. A. COLUMN OF LLOYD'S REAGENT (Plaut, 1963). A column (0.7 cm i.d.) is fitted with a plug of glass wool and filled with a cellulose suspension to a height of bed volume of 2 cm. An aqueous suspension containing 5 g of Lloyd's reagent and 20 g of cellulose powder in 100 ml of water is then applied to a column height of 8 cm. The column is equilibrated with 5% acetic acid. Trichloroacetic acid filtrate (3.00 ml) from the reaction mixture is mixed with 20 ml of 5% acetic acid and applied to the column; 20 ml of 5% acetic acid is applied to remove T_2O. 6,7-Dimethyl-8-ribityllumazine is eluted from the adsorbent with 30 ml of 0.5% pyridine–2% acetic acid. Riboflavin is recovered in the effluent by the addition of 20 ml of 3% pyridine–2% acetic acid.

Essentially complete recovery of radioactivity is made from such columns. Flavin and 6,7-dimethyl-8-ribityllumazine in effluents are measured spectrophotometrically in a Cary Model 14 spectrophotometer in 10-cm light-path cells at 470 and 405 mμ, respectively.

B. THIN-LAYER CHROMATOGRAPHY. The riboflavin containing effluent from the Lloyd's reagent column is concentrated to dryness under reduced pressure in a flash evaporator. The residue is dissolved in water and dried under vacuum again to remove pyridine. The residue is dissolved in 0.6 ml of water and is streaked on a 20 × 19 cm sheet of ChromAR 500 (Mallickrodt). The sheet is dried and developed for 1 hr (ascending) with *n*-butyl alcohol–acetic acid–H_2O (20:3:7.5) in an Eastman Chromagram chamber. This system separates riboflavin (R_F 0.76) from 6,7-dimethyl-8-ribityllumazine (R_F 0.48) and 6-methyl-7-hydroxy-8-ribityllumazine (R_F 0.60).

The riboflavin-containing zone of the sheet is cut out and the flavin is eluted by capillary action with 0.2–0.3 ml of water. The riboflavin content of the eluate is determined spectrophotometrically in 1-cm light-path microcuvet; radioactivity is determined by scintillation counting.

Kuhn–Roth Degradation of Riboflavin. Riboflavin (8–10 mg) is oxidized in chromic acid solution and the acetic acid formed is distilled as described previously (Plaut, 1954). The acetic acid content of the distillate is determined by titration with standard 0.01 N NaOH in a pH meter. The neutralized distillate is evaporated to dryness under vacuum in a flash evaporator to remove T_2O. The residue is made up to volume with water and the tritium content is determined by counting.

Assay of Enzyme Activity. SINGLE-POINT SPECTROPHOTOMETRIC ASSAY. This assay is used to follow enzyme purification. A final volume of 3.0 ml of a medium containing 50 mM potassium phosphate at pH 7.0, 10 mM $NaHSO_3$, 0.6 mM 6,7-dimethyl-8-ribityllumazine, and enzyme is placed in a test tube and the mixture is incubated for 60 min at 37°. At the start and end of the incubation, 1.00-ml samples are withdrawn and added to 0.50 ml of 15% trichloroacetic acid in 12-ml centrifuge tubes. Coagulated protein is removed by centrifugation; the color of the clear supernatant solution is measured at 470 and 405 mμ in 1-cm light-path cells. Net riboflavin formation is calculated as follows: $[(104.6 \times OD_{470\,m\mu}) - (0.670 \times OD_{405\,m\mu})] \times 1.5$ = nmoles of riboflavin/ml of reaction mixture. The assay range is the amount of enzyme to yield 10–50 nmoles of riboflavin/ml per 1 hr. Specific activity of the enzyme is expressed as nmoles of riboflavin formed/mg of protein per hr at 37°. Enzyme protein is determined by the method of Lowry *et al.* (1951). The concentration of bovine serum albumin used as a reference standard is calculated from its ultraviolet absorption at 280 mμ (Tanford and Roberts, 1952).

Continuous Measurement of Riboflavin Formation. Kinetic analyses accompanying the formation of riboflavin from labeled substrates were monitored continuously in 1.0-cm light-path cells containing 1.00 or 3.00 ml of reaction mixture at 25° in a Gilford Model 240 spectrophotometer. The reaction is initiated by the addition of enzyme to the complete reaction mixture and the light absorption at 470 mμ due to the formation of riboflavin is recorded. A molar extinction of 9468 is used in calculating the formation of riboflavin to correct for substrate consumption. Specific conditions of substrate concentration and buffer composition are cited in the text, tables, and figures.

In 50 mM potassium phosphate–10 mM $NaHSO_3$ at pH 7.2 the reaction rate at 25° is approximately half of that at 37°.

Determination of Radioactivity. Samples are counted in a fluor consisting of 1 part of Triton X-100 (Rohm and Haas) and 2 parts of toluene solution containing 4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. (Greene *et al.*, 1968). Fluor (10 ml) is used per vial with sample volumes ranging between 25 and 100 μl or 500 μl. The radioactivity of the contents of the vials was determined in a Nuclear-Chicago Mark I scintillation counter. The counts were corrected for background radiation and for efficiency by external standardization.

Purification of Riboflavin Synthetase from Yeast. GENERAL PROCEDURES. Ammonium sulfate saturation is calculated by the formulation of Noda and Kuby (1957), the saturated solution being a mixture of 707.4 g of ammonium sulfate and 1 l. of water. Dialysis tubing is washed by boiling in several portions of distilled water.

Steps 1 to 4 below are at room temperature. Lower temperatures are used in later steps as specified in the text. The preparations are kept in the dark or in dim light.

1. EXTRACTION. Red Star active dry yeast (12 kg) is treated as follows: Portions of 375 g of yeast, 2.5 l. of 0.24 M $NaHCO_3$ containing 0.01 M Na_2SO_3, and 25 ml of toluene are mixed at low speed for 1 min in the bowl of a 1-gal Waring Blendor (Model CB 5). The suspensions are combined in a large-capacity plastic vessel and stirred for 15–18 hr with an overhead electric mixer with a stainless steel shaft and propeller.

The mixture is stirred mechanically and brought to pH 5.1 by the slow addition of 3 M acetic acid. This requires about 3 hr for the addition of around 8 l. of acid. After standing

for 1 hr the suspension is placed on a number of 1-gal funnels fitted with 50-cm diameter fluted filter papers (E-D 512) and allowed to filter by gravity overnight.

2. First ammonium sulfate fractionation. Solid ammonium sulfate is added with stirring to the filtrate to a final concentration of 52% saturation. The suspension is kept for 3 hr and then centrifuged at 20° in 1-l. plastic cups in the No. 276 rotor of the International PR-2 centrifuge at 2200 rpm for 15 min. The residue is taken up in 5 l. of a solution of 0.1 M ammonium acetate–0.01 M Na_2SO_3, allowed to stand overnight, and then centrifuged in the GSA or GS-3 rotor of a Sorvall RC2B centrifuge at 8000 rpm for 20 min at 20°. The supernatant fluid is saved (step 2a). The residue is suspended in 1 l. of the above buffer and centrifuged, and the supernatant fluid is retained (step 2b).

3. Second ammonium sulfate step. The supernatant fractions from the previous step (steps 2a and b) are combined and the content of ammonium sulfate is determined by the Nessler method (Johnson, 1949). The solution is brought to 50% saturation of ammonium sulfate by the addition of the solid salt, allowed to stand for 2 hr, and then centrifuged at 20° in the stainless steel inserts of a No. 959 rotor of an International PR-2 centrifuge at 4500 rpm for 15 min. The residue is taken up in 2.5 l. of a solution containing 0.1 M ammonium acetate, 0.01 M Na_2SO_3, and 0.2 mM riboflavin. The solution is clarified by centrifugation after standing overnight. The clear supernatant fluid is retained.

4. Heat step. Solid ammonium sulfate is added to the solution to a final concentration of 21% saturation (0.8 M) as determined by the Nessler method. Samples (1 l.) of the solution are placed in 2-l. suction flasks. The vessel is flushed with nitrogen and closed with a 1-hole silicone rubber stopper fitted with a thermometer whose bulb is immersed in the liquid. The flask is heated with occasional gentle agitation in a boiling-water bath until the solution reaches 55°. The contents are then cooled to 25° in an ice bath. The suspensions from individual treatments are combined and centrifuged at 20° in the 959 rotor of an International PR-2 centrifuge at 4500 rpm for 15 min.

5. Dialysis. The clear solution from the previous step is dialyzed with stirring against 12 l. of the solution of 0.1 M ammonium acetate–0.01 M Na_2SO_3 for 2 to 3 hr at 5°. The dialysis is repeated overnight with fresh buffer.

6. Acetone fractionation. The solution from the previous step is brought to a final concentration of 0.2 M mercaptoethanol (14 ml of mercaptoethanol/l.) and adjusted to pH 8.3 with 8 M NH_4OH. Approximately 1-l. portions of the preparations are placed into a 4-l. glass bottle fitted with a stirrer and chilled to −10° in an ethylene glycol bath. Acetone is added slowly to a final concentration of 48% (v/v) and the suspension is left at −10° for 1 hr and then centrifuged at −10° in the No. 959 rotor of an International PR-2 centrifuge at 4500 rpm for 15 min. The residue is discarded and the supernatant solution is brought to a final acetone concentration of 70%, permitted to stand for 1 hr at −10° and centrifuged as before. The supernatant fluid containing little activity is discarded, and the residue is placed into 150 ml of 0.1 M ammonium acetate–0.2 M mercaptoethanol at 5°.

The remaining portions from step 5 are fractionated with acetone as described above. The suspensions of the 48–70% acetone fractions and buffer are combined, homogenized in a large Potter–Elvehjem homogenizer (80-ml capacity), and centrifuged at 3° in the GSA rotor of a Sorvall RC2B centrifuge at 8000 rpm for 15 min. The supernatant solution is stored at 3°.

7. Third ammonium sulfate fractionation. The protein concentration of the solution from the previous step is adjusted to about 10 mg/ml by dilution with 0.1 M ammonium acetate–0.2 M mercaptoethanol. Saturated ammonium sulfate solution is added to a final concentration of 37% saturation. The preparation is left for 20 min in an ice bath and then centrifuged at 3°. The supernatant fluid is brought to 53% saturation by addition of saturated ammonium sulfate solution with stirring. The suspension is centrifuged, the supernatant layer is discarded, and the residue is taken up in 30 ml of 0.1 M ammonium acetate–0.2 M mercaptoethanol and clarified by centrifugation in the SS-34 rotor of the Sorvall RC2B centrifuge at 3° at 18,000 rpm for 10 min. The supernatant fluid is stored at 5°.

8. Acidification and freezing–thawing step. Equal volumes of the solution from the previous step and 0.1 M sodium acetate–acetic buffer at pH 5.2 containing 0.2 M mercaptoethanol are mixed and acidified further to pH 5.40 by the addition of 0.1 M sodium acetate–acetic acid buffer at pH 4.8 containing 0.2 M mercaptoethanol; 25-ml portions are placed in 30-ml capacity Corex glass tubes which are then stored in a deep freezer at −90° for 90 min. The frozen preparations are allowed to stand at room temperature for 5 to 10 min and then warmed to room temperature in a water bath. The precipitate formed is removed by centrifugation and the freezing–thawing–centrifugation step is repeated. The supernatant fraction is kept at −90° overnight and thawed and centrifuged in the morning before proceeding to the next step.

9. Dialysis. The protein concentration of the solution from the previous step is adjusted to approximately 10 mg/ml by the addition of 0.1 M sodium acetate–acetic acid buffer at pH 5.2 containing 0.2 M mercaptoethanol. The diluted enzyme solution is dialyzed with stirring against 25 volumes of the above buffer in a 4-l. graduate cylinder at 5° for 2 hr. The dialysis is repeated for 2 hr against fresh buffer. The solution is clarified by centrifugation in the SS-34 rotor of the Sorvall RC2B centrifuge at 5° and 15,000 rpm for 10 min.

10. First ethanol fractionation. The dialyzed enzyme is warmed to room temperature and brought to 7% (v/v) alcohol by the addition with stirring of 95% ethanol. A blanket of nitrogen is placed above the enzyme solution, and the container is stoppered and incubated at 24° for 4 hr. A white precipitate is removed by centrifugation at 20°. The and supernatant fluid is incubated at 24° overnight as above clarified by centrifugation (step 10a).

The supernatant solution from step 10a is adjusted to 30% ethanol with stirring at −3°. After 2 hr at −3° the suspension is centrifuged at −10° in the SS-34 rotor of the Sorvall RC2B centrifuge at 15,000 rpm for 15 min. The supernatant solution usually contains little activity and is discarded.[2] The residue is taken up in 30 ml of 0.1 M sodium acetate–acetic acid

[2] With certain batches considerable activity has been found in the fraction above 30% ethanol. This activity can be recovered by increasing the alcohol concentration to 40% and allowing the solution to stand overnight at −3°. The precipitate is collected by centrifugation and dissolved in 6 ml of the acetate–mercaptoethanol buffer.

TABLE I: Purification of Riboflavin Synthetase from Yeast.[a]

Step	Vol, ml	Total Protein, mg	Total Act., nmoles of B_2	Activity Yield, %	Sp Act., nmoles of B_2/mg
Extract	65,500	1,315,000	4,780,000	100	3.6
First ammonium sulfate	7,355	317,000	4,800,000	100	15.1
Second ammonium sulfate	3,070	204,000	4,800,000	100	23.5
Heat treatment	2,815	149,000	4,270,000	89	28.7
Dialysis	3,500	136,000	3,720,000	78	27.4
Acetone fractionation	390	14,900	3,000,000	63	201
Third ammonium sulfate	53	4,000	2,890,000	60	723
Acidification and freeze–thawing step	101	2,500	2,490,000	52	996
Dialysis	218	2,300	2,433,000	51	1,058
First ethanol fractionation	42	1,400	2,136,000	45	1,526
Second freezing–thawing step	135	564	1,636,000	34	2,900
Second ethanol fractionation	8.7	249	1,527,000	32	6,133
TEAE-cellulose chromatography	44.6		1,399,000	29	
Concentrate	7.5	104	1,446,000	30	13,900

[a] Active dry yeast, 12 kg.

buffer at pH 5.2 containing 0.2 M mercaptoethanol and clarified by centrifugation (step 10b).

11. SECOND FREEZING–THAWING STEP. The solution from step 10b is diluted with 0.1 M sodium acetate–acetic acid buffer at pH 5.2 containing 0.2 M mercaptoethanol to a protein concentration of 10 mg/ml. This solution is placed in 30-ml capacity Corex glass tubes and placed at −90° for 90 min. The white precipitate formed after thawing is removed by centrifugation, and the supernatant fluid is frozen overnight at −90°, thawed, and centrifuged. The supernatant fluid is retained.

12. SECOND ETHANOL FRACTIONATION. The solution is made 7% in ethanol by the dropwise addition of 95% ethanol at −3°. The precipitate which may form after 1 hr standing at this temperature is removed by centrifugation. The solution is then adjusted to 20% ethanol, kept for 1 hr at −3°, and then centrifuged at −10°. The residue is dissolved in 10 ml of 0.02 M potassium phosphate–0.2 M mercaptoethanol–0.001 M dithioerythritol at pH 7.2 and stored in an ice bath.

13. TEAE-CELLULOSE CHROMATOGRAPHY. A 2 × 4 cm column of TEAE-cellulose is prepared and equilibrated at 5° with 300 ml of 0.02 M potassium phosphate–0.2 M mercaptoethanol–0.001 M dithioerythritol at pH 7.2. The clear solution from the previous step containing 200–300 mg of protein is placed on the column which is then washed with 300 ml of 0.04 M potassium phosphate–0.2 M mercaptoethanol–0.001 M dithioerythritol at pH 7.2. The effluent of a pale yellow color has little activity and is discarded. The enzyme activity and the accompanying yellow color are brought off the column with 0.1 M potassium phosphate–0.2 M mercaptoethanol–0.001 M dithioerythritol at pH 7.2 at a flow rate of 3–4 ml/min.

The fractions containing activity are combined (40–50 ml) and concentrated under vacuum in an S & S collodion membrane filtration apparatus to a final volume of 7 to 8 ml. The collodion membrane is bathed in a solution of 0.1 M potassium phosphate–0.2 M mercaptoethanol–0.001 M dithioerythritol at pH 7.2 during vacuum filtration. The final enzyme preparation is stored in liquid nitrogen.

A typical purification procedure is described in Table I.

Properties of the enzyme. The present method of enzyme purification is a modification of that reported previously (Harvey and Plaut, 1966). It yields a final product of higher specific activity, operational aspects of some of the steps have been simplified, and the yield of activity has been improved 30- to 40-fold.

Examination of an enzyme preparation, specific activity of 13,800 nmoles of riboflavin formed per mg of protein at 37°, by electrophoresis on cellulose acetate strips (0.1 M Tris-HCl–0.01 M Na_2SO_3 buffer at pH 8.5, 250 V for 30 min at 3–5 mA in a Beckman Microzone apparatus) shows a single band of protein containing the activity which migrates toward the anode. Preliminary examination by disc electrophoresis (Davis, 1964) also shows a single band of protein containing the activity. However, additional criteria are needed to determine whether the enzyme has reached the ultimate state of purification.

Results

Deuterium-Labeled 6,7-Dimethyl-8-ribityllumazine 6-Methyl-7-deuteriomethyl-8-ribityllumazine. The enzymic formation of riboflavin from freshly prepared 6-methyl-7-deuteriomethyl-8-ribityllumazine is slower than that from the unlabeled substrates (Figure 2a, b). The ratio of maximal velocities, calculated from initial reaction rates of the experiment in

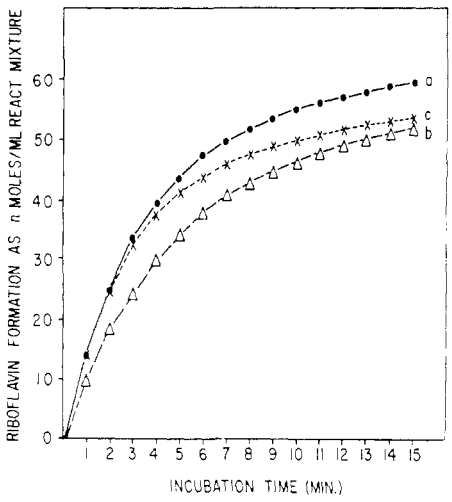

FIGURE 2: Enzymic conversion of 6-methyl-7-deuteriomethyl-8-ribityllumazine and unlabeled substrate into riboflavin. The labeled (7.53 mM) and unlabeled (8 mM) substrates, dissolved in 0.1 M potassium phosphate at pH 6.8, were tested immediately or incubated at 25° for 178 min without enzyme. A 0.01-ml sample was then added to 0.49 ml of reaction mixture containing 50 mM potassium phosphate–10 mM Na_2SO_3 at pH 6.8 and 195 μg of enzyme protein (specific activity 13,800); the formation of riboflavin was measured spectrophotometrically at 25°: (a) (●—●) 6,7-dimethyl-8-ribityllumazine either dissolved freshly or preincubated for 178 min; (b) (Δ——Δ), 6-methyl-7-deuteriomethyl-8-ribityllumazine freshly dissolved; (c) (x---x) 6-methyl-7-deuteriomethyl-8-ribityllumazine preincubated for 178 min.

Figure 2 and assuming K_m (13 μM) for the labeled and unlabeled substrates to be identical,[3] is V_{max} (labeled substrate)/V_{max} (unlabeled substrate) = 0.7–0.8.

The decreased rate with the 7-deuterio compound is not due to inhibitory decomposition products or inhibiting substances in the sample, since preincubation of 6-methyl-7-deuteriomethyl-8-ribityllumazine without enzyme partially restores the rate of enzymic riboflavin formation (Figure 2c). Calculating from a first-order rate constant of k = 0.00465 min^{-1} for the nonenzymic exchange (see Table II), 56% of the deuterium of the 7-methyl group of the lumazine would be replaced by protons under the conditions of preincubation. Riboflavin formation from unlabeled and labeled preincubated substrates is essentially identical during the first 3 min of incubation, during which about 40% of the substrate is consumed (Figure 2a *vs.* c). The slower rate of riboflavin formation in the subsequent incubation period may be due merely to the relative enrichment of deuterium-containing lumazine or, in addition, to inhibition by deuterated lumazine of the transformation of the unlabeled substrate to flavin. The experiment shown in Figure 2 and other experiments varying the conditions of preincubation show that inhibition is reversible at rates commensurate with the rate of nonenzymic replacement of deuterium by protons in the substrate. The presence of deuterium in the 7-methyl group of the substrate is, therefore, responsible for the slower rate of the enzyme-catalyzed reaction.

6-Deuteriomethyl-7-methyl-8-ribityllumazine. Analysis by nuclear magnetic resonance spectroscopy has shown no nonenzymic exchange of deuterium and hydrogen from and into water at the methyl group of position 6 of the lumazine (Beach and Plaut, 1970). However, the rate of enzymic formation of riboflavin from 6-deuteriomethyl-7-methyl-8-ribityllumazine is only about one-fifth of that from the nonlabeled lumazine (Table III). Nonenzymic preincubation of 6-deuteriomethyl-7-methyl-8-ribityllumazine in water under conditions which lead to a rapid hydrogen–deuterium exchange at the 7-methyl group of the lumazine, does not lead to an enhanced enzymic conversion of the 6-deuteriomethyl derivative into riboflavin. Thus, preincubation of 6-methyl-7-deuteriomethyl-8-ribityllumazine in acetate buffer (pH 4.5 at 23°) leads to an increase in the rate of riboflavin formation with time; however, this treatment even for 22.5 hr does not increase the rate with the 6-methyl-labeled lumazine (Figure 3).

Analysis of the kinetics of the enzyme catalyzed reaction with 6-deuteriomethyl-7-methyl-8-ribityllumazine revealed that although the maximal velocity is lower with the labeled than with unlabeled substrate, the values of K_m are essentially identical (Table III). In the presence of several fixed levels of 6-deuteriomethyl-7-methyl-8-ribityllumazine and varying concentrations of 6,7-dimethyl-8-ribityllumazine the rate of formation of riboflavin is lower than with the unlabeled substrate alone (Figure 4A). A plot of the reciprocals of velocity of flavin formation from unlabeled lumazine against 6,7-dimethyl-8-ribityllumazine concentration, corrected for the amount of flavin produced in the presence of 6-deuteriomethyl-7-methyl-8-ribityllumazine alone, suggests competitive inhibition by the 6-deuteriomethyllumazine of enzymic synthesis of riboflavin from the unlabeled substrate (Figure 4B). Close agreement of k_i (12.4 μM) and k_m (13.2 μM) for 6-deuteriomethyl-7-methyl-8-ribityllumazine, calculated from the data in Figure 4B and Table III, respectively, also support a competitive type of inhibition.

TABLE II: Nonenzymic Exchange between 7-Tritiomethyl and 7-Deuteriomethyl Group of 6,7-Dimethyl-8-ribityllumazine and H_2O.[a]

Buffer (M)	pH	Exchange Reaction, First-Order Rate Constant × 10^3 (min^{-1})	
		Tritiated	Deuterated
Sodium acetate–acetic acid (0.1)	4.5	0.23	1.2–3.5
Potassium phosphate (0.1)	5.8	5.5	46
Potassium phosphate (0.05)	6.8	14.9	47
Imidazole-HCl (0.05)	6.8	0.53	3

[a] Incorporation of tritium into water was determined at 25° as described under Experimental Procedures. The exchange between 6-methyl-7-deuteriomethyl-8-ribityllumazine and H_2O was determined at 23° by measuring the change in peak height of the compound by nuclear magnetic resonance spectroscopy as reported previously (Beach and Plaut, 1970).

[3] Preliminary experiments have shown that this assumption is justified.

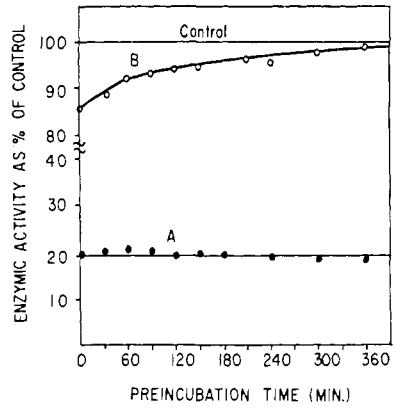

FIGURE 3: Effect of preincubation of deuterated 6,7-dimethyl-8-ribityllumazine on the rates of enzymic riboflavin formation. The substrates were incubated in 0.1 M sodium acetate–acetic acid at pH 4.5 at 25° in the absence of enzyme. Aliquots were withdrawn at time intervals indicated, added to reaction mixtures at pH 6.8 containing enzyme, and the initial velocities of riboflavin formation were determined. The rates of formation of riboflavin from the labeled substrates were compared with those of unlabeled 6,7-dimethyl-8-ribityllumazine (control) preincubated under identical conditions (100%): (A) 6-deuteriomethyl-7-methyl-8-ribityllumazine (0.15 mM); (B) 6-methyl-7-deuteriomethyl-8-ribityllumazine (0.15 mM).

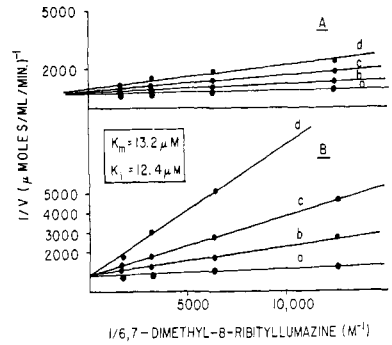

FIGURE 4: Inhibition by 6-deuteriomethyl-7-methyl-8-ribityllumazine of riboflavin formation from 6,7-dimethyl-8-ribityllumazine. Levels of 6-deuteriomethyl-7-methyl-8-ribityllumazine at a, b, c, and d are 0, 0.1, 0.31, and 0.61 mM, respectively. The reaction mixtures containing 0.05 M potassium phosphate at pH 7.2, 13.2 μg/ml of enzyme protein (specific activity 11,700) and the lumazine derivatives were incubated at 25°. Initial velocities of riboflavin formation were determined spectrophotometrically: (A) formation of riboflavin uncorrected for flavin production from 6-deuteriomethyl-7-methyl-8-ribityllumazine; (B) net formation of riboflavin from 6,7-dimethyl-8-ribtyllumazine.

The effect of the 6-deuteriomethyllumazine is not due to inhibition by the deuterated product since similar inhibition was obtained with unlabeled riboflavin and with riboflavin formed from 6-deuteriomethyl-7-methyl-8-ribityllumazine (Table IV).

Tritium-Labeled 6,7-Dimethyl-8-ribityllumazine. The experiments with deuterium-labeled substrates indicate that removal of a deuteron from the 6-methyl group is more rate limiting than that from the 7-methyl group of the lumazine in enzymatic riboflavin formation. Experiments were then conducted with tritiated substrates, in order to follow the distribution of hydrogen isotopes from selectively labeled methyl groups of the substrate into riboflavin and water.

In comparing results with the deuterated and tritiated substrates one must recognize certain basic operational differences. The deuterated compounds contain more than 90% of the hydrogen content of the labeled methyl groups as deuterium. The tritiated substrates, synthesized chemically from water at a specific radioactivity of 100 mCi/ml, contained on an average of only one atom of tritium per 10^5 molecules of substrate. As a result, the selectively labeled methyl groups contain principally three atoms of deuterium, while with the analogous tritiated substrates most of the molecules present are unlabeled; the labeled ones are likely to contain predominantly one atom of tritium and two of hydrogen per methyl group.

6-Tritiomethyl-7-methyl-8-ribityllumazine. The reaction of the labeled lumazine was examined under several conditions

TABLE III: Comparison of k_m and V_{max} of 6,7-Dimethyl-8-ribityllumazine and 6-Deuteriomethyl-7-methyl-8-ribityllumazine.[a]

Compound	k_m, μM	V_{max}, nmoles of B_2/mg of protein per hr
6,7-Dimethyl-8-ribityllumazine	15	5100
6-Deuteriomethyl-7-methyl-8-ribityllumazine	13	1100

[a] The cuvet with variable quantities of 6,7-dimethyl-8-ribityllumazine contained 4.6 μg of enzyme protein (specific activity 13,800) per ml; with 6-deuteriomethyl-7-methyl-8-ribityllumazine, 23.2 μg/ml of enzyme protein was used. The reaction mixtures (50 mM potassium phosphate–10 mM Na_2SO_3 at pH 7.2) were incubated at 25° and the initial rates of riboflavin formation were determined spectrophotometrically at 470 mμ.

TABLE IV: Product Inhibition by Unlabeled and Deuterated Riboflavin.[a]

Product Inhibitor Added	Initial Reaction Rate, nmoles of B_2/min per ml	Inhibition, %
None	1.2	
0.0153 mM Riboflavin	0.95	21
0.0153 mM Deuterated riboflavin[b]	0.87	28

[a] Reaction mixtures contained 0.061 mM 6,7-dimethyl-8-ribityllumazine, 50 mM potassium phosphate–10 mM Na_2SO_3 at pH 7.2, and 13.2 μg of enzyme protein (specific activity 11,700) per ml. Incubation was at 25° and riboflavin formation was determined spectrophotometrically at 470 mμ. [b] Formed enzymically from 6-deuteriomethyl-7-methyl-8-ribityllumazine.

TABLE V: Enzymic Incorporation of Tritium into Riboflavin and Water from 6-Tritiomethyl-7-methyl-8-ribityllumazine.[a]

Compound	Amount, μmoles/ml	Radioactivity, dpm/ml	Specific Radioactivity, dpm/μmoles
6,7-Dimethyl-8-ribityllumazine (initial)	0.626	246,000	393,000
6,7-Dimethyl-8-ribityllumazine (recovered)	0.290	110,000	384,000
Riboflavin (recovered)	0.166	114,000	687,000
Water (net enzymic formation)		11,300	

[a] 6-Tritiomethyl-7-methyl-8-ribityllumazine was dissolved in 50 mM potassium phosphate at pH 7.2. The tritium content of water was determined in a zero time sample. One portion of the lumazine solution was mixed with 139 μg of enzyme protein (specific activity 11,700) per ml and incubated for 14 min at 25°. The second portion of the lumazine solution was incubated without enzyme. In the absence of enzyme no additional tritium was released into water; radioactivity of the initial lumazine and of water recovered after incubation with enzyme have been corrected for tritium in the water of the reaction mixture (20,900 dpm/ml). Riboflavin and 6,7-dimethyl-8-ribityllumazine were separated as described under Experimental Procedures and determined spectrophotometrically.

of incubation varying in enzyme concentration, pH, and time. In all cases, tritium recovered in water after incubation is substantially less than would be expected from the stoichiometry of the conversion of the lumazine into riboflavin (Table V). Furthermore, the molar specific radioactivity of riboflavin recovered is 1.7 to 1.9 times higher than that of the initial 6-tritiomethyl-7-methyl-8-ribityllumazine. Assuming that (a) the labeled compound contains predominantly one atom of tritium per 6-methyl group, (b) the transfer of the 4-carbon moiety forming carbon atoms 6 and 7 and the methyl groups of riboflavin occurs without substantial loss of protons and tritions, and (c) a random loss of protons and tritions from the 6-methyl group of the lumazine which becomes carbon 5 of riboflavin, the ratio of molar specific radioactivities of riboflavin (formed):6-tritiomethyl-7-methyl-8-ribityllumazine should be 1.33. However, isotope discrimination against the removal of tritium from the 6-methyl group would lead to little loss of tritium into water and retention of tritium in riboflavin recovered, with a ratio of molar specific radioactivities of product:substrate approaching 2. The latter is consistent with the experimental results obtained (Table V) and with the severe rate-limiting effect noted with 6-methyl-deuterium-labeled lumazine (Table III).

6-Methyl-7-tritiomethyl-8-ribityllumazine. A nonenzymic exchange of deuterons from and into water at the 7-methyl group of a number of lumazine derivatives has been noted (Beach and Plaut, 1970). The rate of the nonenzymic exchange with 6-methyl-7-tritiomethyl-8-ribityllumazine was further studied to evaluate its role in the riboflavin synthetase reaction. First order rate constants for the exchange reaction with 6-methyl-7-tritiomethyl-8-ribityllumazine under different conditions of pH and buffer composition and comparison of the rate constants of the tritiated compounds with the deuterated substances are given in Table II. Previous studies have shown (Beach and Plaut, 1970) that pH affects the rate of the exchange reaction with the deuterated compounds. The data in Table II demonstrate that the nature of the buffer also has a marked effect on rate of exchange. Thus, at pH 6.8 the rate of nonenzymic loss of tritium from 6-methyl-7-tritiomethyl-8-ribityllumazine is 28 times faster in 0.05 M potassium phosphate than in 0.05 M imidazole-HCl buffer.

In the presence of a relatively large amount of enzyme, the transformation of 6-methyl-7-tritiomethyl-8-ribityllumazine leads to substantial recovery of tritium in water and flavin (Table VI). The quantity of tritium found in water is considerably larger than expected from the nonenzymic exchange with water as calculated from the *initial* concentrations of 6-methyl-7-tritiomethyl-8-ribityllumazine (Table VI, expt 1 and 2). The contribution of enzyme to trition liberation is more apparent in imidazole-HCl buffer (Table VI, expt 2), since the nonenzymic exchange is lower than in phosphate buffer (Table VI, expt 1).

The results obtained with tritiated compounds (Tables V and VI) reveal that 6-methyl-7-tritiomethyl-8-ribityllumazine leads to relatively more tritium in water and less in flavin than the 6-tritiomethyl compound. These results are consistent with the previous observation that deuterium in the 7-methyl group is less rate limiting than in the 6-methyl group of the lumazine (Figure 2 and Table III).

The distribution of tritium from 6-methyl-7-tritiomethyl-8-ribityllumazine into water and riboflavin (Table VI) indicates little or no isotope discrimination between hydrogen and tritium at the 7-methyl group. To illustrate, if one assumes *no* isotope discrimination in the removal of the two protons and a single trition from the 7-methyl group and transfer of the 4-carbon moiety occurs without loss of tritium (Figure 5A), then the ratio of molar specific radioactivities of riboflavin:6-methyl-7-tritiomethyl-8-ribityllumazine should be 1.33, and the ratio of the amounts of tritium recovered in riboflavin:water should be 2. In contrast, if there were absolute discrimination against the removal of tritium from this group, only protons and no tritium ions would be recovered in water and the ratio of molar specific radioactivity of product:substrate should approach 2 (Figure5 B). In the experiments reported in Table VI the ratios of molar specific radioactivities are 1.36 and 1.40, and those of the ratio of amounts of tritium recovered in riboflavin:water were 1.84 and 1.84 (expt 1 and 2, respectively).

Additional support for the scheme involving little isotope discrimination between tritium and hydrogen (Figure 5A) comes from studies of first-order rate constants for formation of riboflavin and the accompanying tritium incorporation into water, and from the distribution of tritium in riboflavin formed from 6-methyl-7-tritiomethyl-8-ribityllumazine. These reactions followed pseudo-first-order kinetics (Table VII). Comparable values of the rate constants for both reactions were obtained when calculated from the stoichiometry of

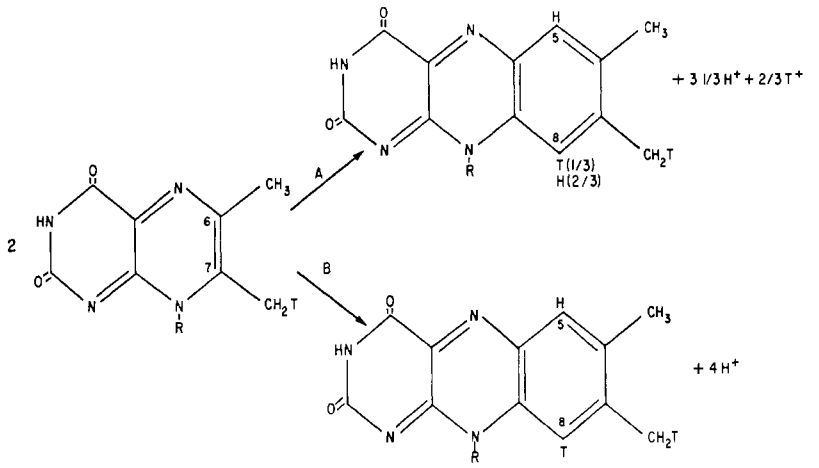

FIGURE 5: Distribution of tritium from 6-methyl-7-tritiomethyl-8-ribityllumazine into riboflavin and water in the absence (A) and presence (B) of isotope discrimination.

TABLE VI: Incorporation of Tritium into Riboflavin and Water from 6-Methyl-7-tritiomethyl-8-ribityllumazine.[a]

Expt	Compound	Amount (A), μmoles/ml	Enzymic Radioactivity (B), dpm/ml	Enzymic Radioactivity (B/A), Specific dpm/μmole	Nonenzymic Radioactivity[b] (C), dpm/ml
1	6-Methyl-7-tritiomethyl-8-ribityllumazine (initial)	0.1568	59,060[c]	376,700	59,060
	Water (5 min)		11,500[c]		4,000
	Water (10 min)		14,400[c]		7,730
	Riboflavin (5 min)	0.0435			
	Riboflavin (10 min)	0.0516	26,520	513,900	
2	6-Methyl-7-tritiomethyl-8-ribityllumazine (initial)	0.1471	57,400[c]	390,200	57,400
	Water (5 min)		13,000[c]		5
	Water (10 min)		16,140[c]		29
	Riboflavin (10 min)	0.0541	29,680	548,600	

[a] The experimental conditions were similar to those described in Table V. The reaction mixtures contained 0.05 M potassium phosphate at pH 6.8 and 0.05 M imidazole-HCl at pH 6.8, expt 1 and 2, respectively. Enzyme protein (139 μg/ml) (specific activity 13,800) were used in both experiments; in expt 2 the enzyme was dissolved in 0.05 M imidazole-HCl buffer at pH 6.8. Enzyme activities calculated from initial reaction rates were 4520 and 5480 nmoles of riboflavin formed per mg of protein per hr at 25° in expt 1 and 2, respectively. [b] The incorporation of tritium into water at 5 and 10 min was calculated from first-order rate constants [14.9×10^{-3} and 0.53×10^{-3} min^{-1} for reaction mixtures containing 0.05 M potassium phosphate at pH 6.8 (expt 1) and 0.05 M imidazole-HCl at pH 6.8 (expt 2), respectively] from the net radioactivity of 6-methyl-7-tritiomethyl-8-ribityllumazine added initially. [c] Corrected for radioactivity present in water at zero time; 17,500 and 10,500 dpm per ml in expt 1 and 2, respectively.

riboflavin synthesis (Figure 1) and tritium incorporation into water (Figure 5A). The agreement of the rate constants for riboflavin formation and trition liberation into water is consistent with the findings reported in Table VI; however, the experimental procedures used (Table VII) are probably inherently more reliable since observations could be made at several incubation times and they do not depend on the technically more difficult task of recovering riboflavin quantitatively. Partial chemical degradation by the Kuhn–Roth method of riboflavin formed from the 7-tritiomethyl-lumazine yields carbon atoms 6 and 7 and the attached methyl groups of riboflavin as acetic acid (Plaut, 1954). The mechanism of Figure 5A would lead to ratios of specific molar activities of 6-methyl-7-tritiomethyl-8-ribityllumazine:acetate and acetate:riboflavin of 2.00 and 0.375, respectively. This compares to experimental values of these ratios of 2.17 and 0.36, respectively (Table VIII).

Enzyme-Catalyzed Exchange between Water and the 7-Tritiomethyl Group. The experiments above show the elimination of tritions into water proceeding at a rate equivalent to that

TABLE VII: Comparison of First-Order Rate Constants for the Enzymic Formation of Riboflavin and Incorporation of Tritium into Water from 6-Methyl-7-tritiomethyl-8-ribityllumazine.[a]

Expt	A, Riboflavin Formation First-Order Rate Constant, $k \times 10^3$ min^{-1}	B, Tritium Incorporation into H_2O, First-Order Rate Constant, $k \times 10^3$ min^{-1}	A:B
1	73.0	72.0	1.01
2	21.4	20.9	1.02

[a] The reaction mixtures contained 0.1 M imidazole-HCl at pH 6.8, 0.15 mM 6-methyl-7-tritiomethyl-8-ribityllumazine with 40.6 μg and 15 μg of enzyme protein per ml of reaction mixture in expt 1 and 2, respectively. Riboflavin formation at 25° was recorded continuously at 470 mμ and tritium in water was determined as described under Experimental Procedures. Samples were withdrawn at 0-, 5-, 10-, 15-, and 20-min intervals. First-order rate constants, $k = 2.303/t \log (a/(a - x))$ were calculated from values at varying time intervals (t), where in the case of riboflavin formation: a = [6,7-dimethyl-8-ribityllumazine (initial)] and $x = 2 \times$ [riboflavin] while for tritium incorporation into water: a = dpm/ml of reaction mixture in 6-methyl-7-tritiomethyl-8-ribityllumazine (initial) and $x = 3$ [dpm/ml of reaction mixture in water (corrected for zero time)].

TABLE IX: Exchange Reaction between the 7-Tritiomethyl Group of Various Lumazine Derivatives and Water in the Presence and Absence of Enzyme.[a]

Compound	Exchange Reaction, First-Order Rate Constant, $k_{app} \times 10^3$ min^{-1}[b] Enzyme Present	Enzyme Absent
6,7-Dimethyl-8-ribityllumazine	33.0	0.53
6-Deuteromethyl-7-methyl-8-D-ribityllumazine	26.5	1.2
6,7-Dimethyl-8-[1′-(5′-deoxy-D-ribityl)]lumazine	12.9	0.98
6,7-Dimethyl-8-(D-xylityl)lumazine	8.9	1.25
6,7-Dimethyl-8-(L-xylityl)lumazine	1.7	1.33

[a] All incubations were carried out in 0.05 M imidazole-HCl at pH 6.8 and 25°. Concentrations of lumazine derivatives containing 7-tritiomethyl groups were between 0.104 and 0.158 mM. Enzyme, when present, was at 194 μg/ml of enzyme protein (specific activity 13,800). Tritium content of water was analyzed as described under Experimental Procedures. [b] $k_{app} = 2.303/t \log (a/(a - x))$, where a = dpm/ml of reaction mixture in 7-tritiomethyllumazine derivative (initial) and x = dpm/ml of reaction mixture in water at varying time intervals (t). All samples were corrected for the tritium content of water at zero time.

TABLE VIII: Partial Chemical Degradation of Riboflavin Formed from 6-Methyl-7-tritiomethyl-8-ribityllumazine.[a]

Compound	Specific Radioactivity dpm/μmole	Ratio
6,7-Dimethyl-8-ribityllumazine (initial)	479,000	
Riboflavin (formed)	611,000	
Acetic acid (degradation product of riboflavin)	221,000[b]	
Riboflavin–6,7-dimethyl-8-ribityllumazine		1.28
6,7-Dimethyl-8-ribityllumazine–acetic acid		2.17
Acetic acid–riboflavin		0.36

[a] Riboflavin was formed enzymically under conditions similar to those described in Table VI (expt 2). Flavin was separated and purified from the reaction mixture by chromatography first on a column of Lloyd's reagent followed by thin-layer chromatography on ChromAR 500 as described under Experimental Procedures. [b] After purification, riboflavin was diluted with a 200-fold excess of unlabeled substance before chromic acid oxidation. The specific radioactivity of the acetic acid formed has been corrected for dilution.

of riboflavin formation. It seemed possible that under other conditions an enzyme-catalyzed exchange between the 7-tritiomethyl group and water could be shown to proceed at a rate different from that of the overall reaction. At pH 4.5 in acetate buffer and at pH 5.8 in phosphate buffer, where riboflavin formation is absent or markedly lower than at the optimal pH (Harvey and Plaut, 1966) no significant enhancement of the exchange due to the presence of enzyme could be observed.

This observation may suggest that the nonenzymic hydrogen–tritium exchange, reflecting a structural change in the lumazine molecule to a form which serves as the substrate for the enzyme, may be fast enough to accommodate the subsequent possible pH-dependent enzymic transformation(s). However, this seems unlikely since in the presence of enzyme, the formation of riboflavin and elimination of tritions occurs at a faster rate than the nonenzymic exchange (Table VI). Furthermore, the velocity of the riboflavin synthetase reaction at pH 6.8 is about 25% more rapid in 0.05 M imidazole-HCl than in 0.05 M potassium phosphate buffer, while the nonenzymic exchange proceeds much more rapidly in phosphate than in imidazole buffer (Table II).

Alternatively, elimination of protons from the 7-methyl group of the lumazine may be an irreversible step in the enzymic transformation to riboflavin, or an initial reversible step leading to elimination of protons is followed by an irreversible transformation proceeding at the same or faster rate. Elimination of protons from the 7-methyl group of the lumazine should be stoichiometric with riboflavin formation

TABLE X: The Incorporation of Tritium into Water and Flavin from the 7-Tritiomethyl Groups of 6,7-Dimethyl-8-(5′-deoxyribityl)-lumazine and 6-Deuteriomethyl-7-methyl-8-ribityllumazine.[a]

Compound	Incubation Time, min	Radioactivity with Enzyme Present: (A) Amount, μmoles/ml	(B), dpm/ml	(B/A) Specific, dpm/μmole	Radioactivity with Enzyme Absent: (C), dpm/ml
6,7-Dimethyl-8-(5′-deoxy-D-ribityl)lumazine	0	0.1035	148,670[b]	1,436,000	148,670
5′-Deoxyriboflavin	60	0.0127	11,200	881,000	
Water	60		80,750[b]		8,470[c]
6-Deuteriomethyl-7-methyl-8-ribityllumazine	0	0.1583	77,400[d]	489,000	77,400
Riboflavin	30	0.0589	24,270	412,000	
Water	30		43,300[d]		2,690[c]

[a] The experimental conditions were similar to those described in Table VI (expt 2) excepting the length of incubation and the substrates indicated. Experiments with authentic compounds showed that 6,7-dimethyl-8-(5′-deoxy-D-ribityl)lumazine and 5′-deoxyriboflavin are recovered in fractions analogous to those for the corresponding ribityl derivatives in chromatography on Lloyd's reagent and ChromAR 500; 195 μg/ml of enzyme protein (specific activity 13,800) was added to reaction mixtures with either substrate. [b] Corrected for radioactivity present in water at zero time (36,700 dpm/ml). [c] Calculated from radioactivity present in lumazine initially and first-order rate constants in Table II. [d] Corrected for radioactivity presence in water at zero time (21,600 dpm/ml).

in both mechanisms. An exchange reaction should not occur under any circumstance in the former case; in the latter mechanism eliminating or slowing the second step of the enzyme-catalyzed reaction could unmask the hydrogen exchange at the 7-methyl group of the lumazine.

It seemed possible to determine whether riboflavin synthetase catalyzes such an exchange reaction by use of certain analogs of 6,7-dimethyl-8-ribityllumazine labeled with tritium in the 7-methyl group. These compounds, while bound to the enzyme with about the same affinity, are either converted into flavin at a slower rate than the natural substrate or cannot be transformed at all. The rate of incorporation of tritium into water from the 7-tritiomethyl groups of 6,7-dimethyl-8-[1′-(5′-deoxy-D-ribityl)]lumazine and 6,7-dimethyl-8-(1′-D-xylityl)lumazine is much faster in the presence than in the absence of enzyme (Table IX). Furthermore, 6,7-dimethyl-8-[1′-(5′-deoxy-D-ribityl)]lumazine, which is converted into flavin at about 1% the rate of 6,7-dimethyl-8-ribityllumazine [though the value of k_m for both substrates is similar (Harvey and Plaut, 1966)], leads to substantially larger amounts of tritium in water than in the flavin (Table X). Similar results were obtained with 6-deuteriomethyl-7-tritiomethyl-8-ribityllumazine (Table IX and X). The 6-deuteriomethyl compound has the same k_m but a lower V_{max} than the nonlabeled substrate (Table III). An enzyme-catalyzed exchange between the 7-tritiomethyl group and water also occurs with 6-methyl-7-tritiomethyl-8-D-xylityllumazine (Table IX), a competitive inhibitor which is bound to riboflavin synthetase but not converted into flavin (Harvey and Plaut, 1966; Winestock *et al.*, 1963). Specificity of the riboflavin synthetase and the exchange reaction is supported by the observation that the enzyme does not increase liberation of tritium into water from 6-methyl-7-tritiomethyl-8-L-xylityllumazine (Table IX), a compound which is not an inhibitor of the enzyme or bound to the protein (Harvey and Plaut, 1966; Winestock *et al.*, 1963).

The demonstration of the occurrence of the exchange reaction with 7-tritiomethyl analog at rates faster than flavin formation indicates that proton elimination from the 7-methyl group is an early step in the riboflavin synthetase reaction.

Inhibitors

It was shown in previous studies (Harvey and Plaut, 1966) that yeast riboflavin synthetase activity is inhibited by low concentrations of *p*-mercuribenzenesulfonate. It became of interest to know whether this or other sulfhydryl group binding reagents would also affect the hydrogen exchange reaction between the 7-methyl group of the lumazine substrate and water.

The presence of a concentration of *p*-mercuribenzenesulfonate, which causes 96% inhibition of riboflavin formation, inhibits the incorporation of tritium into water from 6-methyl-7-tritiomethyl-8-ribityllumazine to about the same extent. It should be noted that in this experiment a substantial amount of mercaptoethanol was transferred into the reaction mixture with the enzyme solution. This probably accounts for the relatively high concentration of *p*-mercuribenzenesulfonate required to cause inhibition. In previous experiments, where sulfhydryl compounds were not used to protect the enzyme, 30 μM *p*-mercuribenzenesulfonate resulted in 50% inhibition of activity (Harvey and Plaut, 1966).

5,5′-Dithiobis(2-nitrobenzoate) (Ellman, 1959) has been found to be a potent inhibitor of enzymic riboflavin formation and at the concentration used (50 μM) inhibited the tritium exchange reaction to about the same extent (Table XI). Iodoacetamide causes inhibition of these activities, however,

TABLE XI: The Effect of Various Inhibitors on Enzymic Riboflavin Formation and Tritium Incorporation into Water.[a]

Substance Added	Inhibitor Concn, mM	Inhibition, % Riboflavin Formation	Inhibition, % Tritium Incorporation into Water
p-Mercuribenzenesulfonate[b]	0.3	0	
p-Mercuribenzenesulfonate[b]	3	96	100
5,5′-Dithiobis(2-nitrobenzoate)[c]	0.02	74	
5,5′-Dithiobis(2-nitrobenzoate)[c]	0.05	89	85
Iodoacetamide[d]	0.67	16	
Iodoacetamide[d]	3.35	58	
Iodoacetamide[d]	7.5	71 (21)	75 (24)
Iodoacetamide[d]	20	90 (38)	90 (44)
Iodoacetamide[d]	30	(52)	(52)

[a] All incubations were at 25°. Whenever inhibitors were preincubated with enzyme before addition of substrate to start the reaction the concentrations of inhibitor refer to the preincubation mixture. A control without inhibitor accompanied each preincubation. Incubation in the absence of inhibitor led to no more than 10% loss of enzyme activity. Riboflavin formation was determined spectrophotometrically and the tritium content of water was analyzed as described under Experimental Procedures. Samples for tritium analysis were withdrawn after 0-, 5-, 10-, 15-, and 20-min incubation and riboflavin formation was recorded continuously throughout this period. The inhibition reported represents the average obtained from all time points throughout the incubation period. [b] The reaction mixtures contained 0.05 M potassium phosphate at pH 6.8, 0.148 mM 6-methyl-7-tritiomethyl-8-ribityllumazine, and *p*-mercuribenzenesulfonate as indicated in a total volume of 3.0 ml. The reaction was started by the addition of 194 μg/ml of enzyme protein (specific activity 11,000). The final concentration of sulfhydryl compounds carried into the reaction mixture from the enzyme solution was equivalent to about 2.8 mM mercaptoethanol. [c] Enzyme (144 μg of total protein; specific activity 13,800) and 5,5′-dithiobis(2-nitrobenzoic acid) at the concentrations indicated in 2.95 ml of 0.05 M imidazole-HCl at pH 6.8 were incubated for 90 min at 25°. The reaction was started by the addition of 0.05 ml of 9.3 mM 6-methyl-7-tritiomethyl-8-ribityllumazine. [d] Enzyme (149 μg of protein; specific activity 13,800) in 0.15 ml of 0.1 M imidazole-HCl at pH 6.8 and 0.15 ml of iodoacetamide in 0.1 M imidazole-HCl at pH 6.8 were mixed and incubated for 120 min at 25°. The numbers in parentheses refer to the inhibition at 15-min preincubation. The concentrations of inhibitor refer to those in the preincubation mixtures. The reactions were started by the addition of 2.7 ml of 6-methyl-7-tritiomethyl-8-ribityllumazine (0.16 mM) in 0.05 M imidazole-HCl at pH 6.8.

much larger concentrations of this compound than 5,5′-dithiobis(2-nitrobenzoate) are needed for equivalent inhibition (Table XI). In contrast to *p*-mercuribenzenesulfonate the interaction between iodoacetamide and enzyme appears to be relatively slow. Thus, substantially less inhibition occurs with equivalent concentrations of iodoacetamide when the preincubation period of inhibitor with enzyme is shortened from 120 to 15 min (numbers in parentheses of Table XI).

The results with inhibitors further strengthen the possibility that the exchange reaction and riboflavin formation are catalyzed by the same protein.

Discussion

The results obtained in the present experiments suggest that the enzyme-catalyzed elimination of protons from the 6-methyl and 7-methyl groups of the substrate, which accompanies ring closure to form riboflavin (Figure 1), may occur in two or more sequential steps, probably involving enzyme bound intermediates. Previous experiments showed (Harvey and Plaut, 1966) that riboflavin synthetase contains separate sites binding lumazines, one functioning in donating and the other in accepting the 4-carbon moiety. The present results with 6-methyl-7-tritiomethyl-8-ribityllumazine indicate that the loss of protons occurs from the acceptor molecule of the lumazine, since the methyl groups of riboflavin formed retain essentially all of the tritium which should have been transferred from the donor molecule (Table VIII).

The enzyme-catalyzed enhancement of hydrogen–tritium exchange between the 7-tritiomethyl group of a number of lumazine derivatives and water probably reflects the reversible part of the mechanism involved in the elimination of protons from the 7-methyl group of the substrate. Enzymic enhancement occurs only with analogs of lumazine which lead to formation of flavin (6,7-dimethyl-8-[1′-(5′-deoxy-D-ribityl)]-lumazine and 6-deuteriomethyl-7-methyl-8-ribityllumazine) or which can act as inhibitors of the enzyme (6,7-dimethyl-8-D-xylityllumazine); 6,7-dimethyl-8-L-xylityllumazine, which does not interact with the enzyme, shows no increase in the rate of the exchange reaction (Tables IX and X). It is possible that the enzyme-catalyzed exchange reaction observed with analogs of the substrate may still not be related to the mechanism of formation of riboflavin. It seems noteworthy, however, that the apparent first-order rate constant, k_{app}, for the incorporation of tritium into water from 6-methyl-7-tritiomethyl-8-ribityllumazine is similar in magnitude to those obtained with 6-deuteriomethyl-7-tritiomethyl-8-ribityllumazine and 6-methyl-7-tritiomethyl-8-[1′-(5′-deoxy-D-ribityl)]lumazine even though the rates of flavin formation from the latter analogs are markedly lower than that of the natural nondeuterated substrate (Table IX).

The enzyme-catalyzed elimination of protons at the 7-methyl group probably precedes that at the 6-methyl group. This is consistent with the observation that while the conversion of 6-deuteriomethyl-7-methyl-8-ribityllumazine into riboflavin proceeds at about 20% the rate of the unlabeled substrate (Table III), the velocities of incorporation of tritium into water are similar for 6-deuteriomethyl-7-tritiomethyl-8-ribityllumazine and 6-methyl-7-tritiomethyl-8-ribityllumazine (Table IX). If reaction at the 6-methyl group occurred first the rate-limiting effect of deuterium in the 6-methyl group should also retard tritium–hydrogen exchange

at the 7-methyl group. Furthermore, ring closure to form a flavin does not occur with 6,7-dimethyl-8-D-xylityllumazine; nevertheless, an enzyme-catalyzed hydrogen–tritium exchange at the 7-methyl group of this derivative has been observed (Table IX).

A number of mechanisms can be proposed to explain the reaction. For example, it has been suggested (Rowan and Wood, 1963, 1968) that the chemical transformation of 6,7-dimethyl-8-ribityllumazine to riboflavin involves an opening of the pyrazine ring between nitrogen 8 and carbon 7 initiated by nucleophilic attack (see Beach and Plaut, 1970, Scheme A–C). In a subsequent aldol condensation two molecules of this substance (B) are thought to condense to form a dimeric intermediate (C), with cyclization to riboflavin by a mechanism involving the 6-methyl group and carbon 6 of the initial lumazine. This chemical formation of riboflavin occurred by refluxing the lumazine in aqueous phosphate buffer at neutral pH (Rowan and Wood, 1963, 1968). It may be significant that the present study demonstrates that phosphate enhances the chemical hydrogen–tritium exchange at the 7-methyl group (Table II). Spectrophotometric observations indicate the occurrence of a ring-opening reaction preceded by hydration of the pyrazine portion under alkaline conditions (Pfleiderer *et al.*, 1966), and measurement of nuclear magnetic resonance spectra (Beach and Plaut, 1970) suggest that a compound (B) containing a keto group adjacent to the 7-methyl group is formed from certain lumazine derivatives in alkali. Such an α-methyl ketone would participate readily in hydrogen–tritium (or deuterium) exchange at the methyl group; however, it is unlikely that a mechanism involving compounds A and B (see Beach and Plaut, 1970, Scheme A–C) is applicable in acid solution since the ring-opening reaction and hydration of the pyrazine portion of the lumazine which can be demonstrated under alkaline conditions is readily reversible by acidification (Pfleiderer *et al.*, 1966; Beach and Plaut, 1970). Furthermore, the rate of the chemical exchange of hydrogen at the 7-methyl group of 6,7-dimethyl-8-ribityllumazine and its derivatives is maximal not only in alkali but also in *acid* (Beach and Plaut, 1970) and chemical synthesis of riboflavin from 6,7-dimethyl-8-ribityllumazine occurs in good yield upon heating in 0.1 M HCl under a nitrogen atmosphere (Beach and Plaut, 1969).

It is also doubtful whether the mechanism of Rowan and Wood for the chemical transformation can be used to explain the riboflavin synthetase reaction, although it has not been excluded conclusively. Intermediates such as A and B (see Beach and Plaut, 1970) with opened pyrazine rings should no longer show light absorption at 407 mμ. However, complexes of substrate and enzyme are found to still possess absorption at the long wavelength; the transformation of such complexes to products was followed by continuous scanning of the spectral change and essentially all absorption observed in the visible portion was accounted for by the spectra of 6,7-dimethyl-8-ribityllumazine and riboflavin (Harvey and Plaut, 1966). Experiments with 6-deuteriomethyl-7-methyl-8-ribityllumazine, which has the same k_m but is transformed to flavin at a slower rate than the unlabeled substrate, indicate no intermediate with diminished absorption near 400 mμ.[4]

[4] R. Beach, T. Aogaichi, and G. W. E. Plaut, unpublished observations (1968).

Compounds such as A and B should form derivatives with hydroxylamine; however, addition of the latter in high concentrations did not lead to a reduction in rate of the enzyme-catalyzed reaction (Harvey and Plaut, 1966).

A mechanism for the action of riboflavin synthetase is proposed in Figure 6, which attempts to correlate data from the present and previous studies. It is visualized that binding of substrate to the enzyme occurs at two sites, one leading to donation of the 4-carbon unit (donor site), the other to accept this moiety (acceptor site) (Figure 6, I). Tight binding of the lumazine to what appears to be the donor site occurs over a wide range of pH, from acidic conditions (pH 4.5) where the enzyme is inactive to neutrality, where maximal riboflavin formation is found (Harvey and Plaut, 1966). A hydrophobic region on the protein may be responsible for the relatively pH insensitive binding of the lumazine to this site, also accounting for the bathochromic shifts of absorption maxima in the visible region of the spectrum which accompany binding of lumazine derivatives and riboflavin to the enzyme. If so, then the observed pH dependence of the reaction may be due to interaction at the acceptor site. This has been envisioned to occur (I and II) by hydrogen bonding between YH on the protein and the 2-oxo group of the substrate, facilitating extraction of a proton from the 7-methyl group of the lumazine by a nucleophilic center (A:) on the enzyme. Subsequent carbon–carbon bond formation between the 7-methylene group of the lumazine at the acceptor site and the electrophilic center developed at carbon 7 of the lumazine bound to the donor site (II) results in formation of a dimeric substance (III). If the dissociation constant of group A has a value of pK_a between 6 and 7 it would be protonated under acidic conditions and transformation from I to II would not occur. This is consistent with the observation that neither the enzyme-catalyzed hydrogen exchange at the 7-methyl group of the lumazine nor riboflavin formation proceeds under acidic conditions, but occur at neutrality where flavin synthesis is optimal.

III is visualized to lead to a ring-opened form (IV) which upon protonation generates an electrophilic center at carbon 6 of the lumazine bound to the donor site V. The generation of this electrophilic center facilitates the removal of a proton from the 6-methyl group resulting in structure VI. Ring closure between the 6-methylene group of the lumazine moiety at the acceptor site and the electrophilic center developed at carbon 6 of the lumazine residue at the donor site occurs to form (VII). Finally, a proton is abstracted from VII cleaving the nitrogen–carbon bond resulting in the formation of riboflavin and 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (VIII), the final step being energetically favored since an aromatic resonant system results.

In the scheme outlined (Figure 6) the initial interactions between substrate and enzyme (I and II) envisions tautomeric forms of the bound lumazine, which should retain absorption at long wavelengths. In the subsequent dimeric intermediates (III–VI) opening of the pyrazine ring, possibly resulting in loss of light absorption above 400 mμ, is confined to one of the two lumazine components of the structures. This might be consistent with the failure to find significant reduction in color at around 400 mμ in the enzymic reaction. The absence of an α-methyl ketone group in the proposed intermediates would also be in agreement with the earlier observation that hydroxylamine at high concentrations does not inhibit the

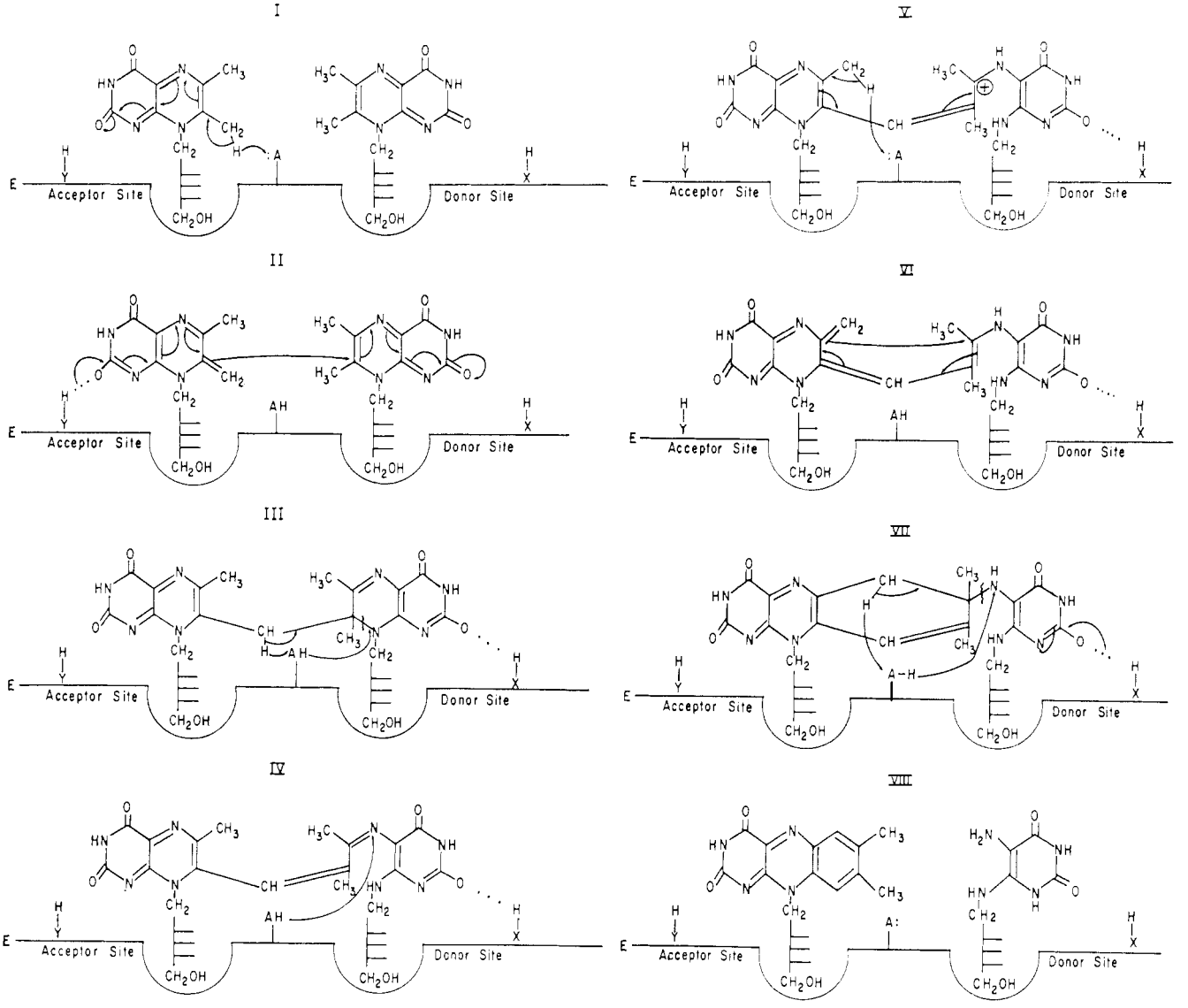

FIGURE 6: Scheme of mechanism of riboflavin synthetase.

reaction (Harvey and Plaut, 1966). Hydrogen exchange at the 7-methyl group could occur in the transformation of I to II and, if the reactions were reversible, between II and IV.

The step which becomes rate limiting upon introduction of deuterium into the 6-methyl group (Table III and Figure 4) may involve the proton abstraction and/or ring-closure reactions pictured between compounds I and VIII.

The nature and number of specific functional groups on the enzyme involved in substrate binding and catalysis is essentially unknown. It may be that certain aromatic amino acids are responsible for binding of substrate and flavin, as has been suggested for the binding of flavin cofactors to flavoproteins (Yagi and Ozawa, 1960; Strittmatter, 1961; Wilson, 1966). If the mechanism proposed here is correct, a group(s) on the enzyme would facilitate the abstraction of protons from the methyl groups of the lumazine at the acceptor site. For the sake of simplicity only a single group (A) playing this role has been shown in the scheme (Figure 6); this is not meant to exclude the possible involvement of more than one such region on the enzyme. In any case, since riboflavin formation and hydrogen exchange at the 7-methyl group of lumazine proceed at about the same rate around neutrality, an imidazole and/or sulfhydryl group(s) may fulfill this function acting preferentially as proton acceptor and donor, respectively.[5] Inhibition of riboflavin formation and hydrogen–tritium exchange at the 7-methyl group of the lumazine by *p*-mercuribenzenesulfonate, iodoacetamide and, particularly, 5,5′-dithiobis(2-nitrobenzoate) (Table XI) makes it likely that a thiol group(s) is involved in the enzyme

[5] If a group on the enzyme with a pK_a of 6–7 (*e.g.*, an imidazole group) facilitates extraction of a proton at an early step (*e.g.*, Figure 6 (I)) while another group with pK_a of 8–9 (a thiol group) acts preferentially as a proton donor at a later step(s) (*e.g.*, Figure 6 (IV)) one might expect that raising the pH above that optimal for riboflavin formation could lead, nevertheless, to an increased exchange rate. Preliminary experiments support this possibility. Thus, the rate of trition elimination from 6-methyl-7-tritiomethyl-8-ribityllumazine is doubled at pH 8.8 while riboflavin production is reduced to 15% of that at the optimum.

action. This relationship is also supported by the previous observation that 6,7-dimethyl-8-ribityllumazine protects the enzyme against inhibition by *p*-mercuribenzenesulfonate and that complexes of the enzyme with riboflavin can be dissociated by low concentrations of the mercurial (Harvey and Plaut, 1966). However, it is at present uncertain whether a sulfhydryl group is located at the catalytic site or at a more remote position on the protein where interaction with thiol reagents may influence enzyme action by steric effects or changes in protein conformation.

In addition to interaction between protein and the heterocyclic rings of the substrate at the donor and acceptor sites, binding of the side chain at position 8 of the lumazine to specific regions on the enzyme would be expected. Previous studies have shown marked specificity of the enzyme for configuration of the side chain in formation of flavin, or inhibition, and in formation of protein–lumazine complexes (Winestock *et al.*, 1963; Harvey and Plaut, 1966). This is in accord with the substrate specificity needed for hydrogen exchange at the 7-methyl group observed here (Table IX). The location of ribityl groups on the enzyme in the scheme (Figure 6) is intended to indicate that the sterically exact attachment of the side chain to the protein may be of importance for the precise positioning of the heterocyclic ring portions at regions of the enzyme which catalyze the transfer of the 4-carbon moiety, leading to product formation.

The sequence of reactions suggested in Figure 6 is in accord with experimental observations. Further characterization of intermediates of the reaction and functional groups of the enzyme is necessary to confirm the scheme.

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