SYNTHESIS OF RIBOFLAVIN BY MICROORGANISMS

V. PROPERTIES OF ENZYME SYSTEM INVOLVED IN THE ENZYMATIC RIBOFLAVIN SYNTHESIS FROM 8-*N*-RIBITYL-6, 7-DIMETHYLLUMAZINE

HIDEO KATAGIRI, ISAO TAKEDA AND KAZUTAMI IMAI

Department of Agricultural Chemistry, Faculty of Agriculture, University of Kyoto, Sakyo, Kyoto

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In the preceding paper (1), the authors showed that 8-*N*-ribityl 6,7-dimethyl-lumazine (6,7-dimethylribolumazine) isolated both from mycelia of *Eremothecium ashbyii* and from culture filtrate of *Clostridium acetobutylicum* might be one of the direct precursors of the riboflavin biosynthesis. In addition, it was suggested that acetate might be an important substance among the 4-C-donors which are required for enzymatic conversion of 6,7-dimethylribolumazine to riboflavin. The enzymatic formation of riboflavin from 6,7-dimethylribolumazine was also found with *Er. ashbyii* as well as beef liver. The enzyme involved in this reaction system seems to be of a wide distribution and of a relatively nonspecific nature.

The authors found, furthermore, that the partially purified enzyme required cofactors for the reaction. In the present study, a series of experiments were performed to elucidate the properties of this enzyme and to ascertain the natures of its cofactors.

EXPERIMENTAL

1. Microorganisms

Cl. acetobutylicum W-29, *E. coli neapolitanus* B-25, *Er. ashbyii* Guilliermond 0557, *Neurospora crassa*, *Lactobacillus plantarum* and *Lactobacillus casei* were used in the present research. *Er. ashbyii* was given from the Institute for Fermentation, Osaka.

2. Materials

6,7-Dimethylribolumazine was kindly given from the Research Laboratory of Takeda Pharmaceutical Industries Ltd., Osaka. CoA¹ was the commercial product of Nutritional

¹ The following abbreviations are used: Coenzyme A, CoA; acetyl coenzyme A, acetyl CoA; adenosine triphosphate, ATP; diphosphopyridine nucleotide, DPN; reduced diphosphopyridine nucleotide, DPNH; triphosphopyridine nucleotide, TPN; reduced triphosphopyridine nucleotide, TPNH; thiamine pyrophosphate, TPP; acetyl phosphate, acetyl-P; tris-(hydroxymethyl)-aminomethane, Tris; disodiumethylenediamine tetracetate, EDTA; glutathione, GSH.

Biochemicals Corporation, Cleveland. ATP¹ was of Zellstofffabrik Waldhof, Germany. DPN¹ and TPN¹ of Sigma Chemical Company, St. Louis, and TPP¹ of L. Light and Company Ltd, Poyle, England (2). DPNH¹ and TPNH¹ were prepared according to Lepage (2). Dowex resin No. 1 was of Dow Chemical Company, Midland and Amberlite IRA-400 of Rohm and Haas Company, Philadelphia. Acetate, acetaldehyde and diacetyl were the commercial products. Acetyl-P¹ was prepared by the method of Avison (3).

3. Assay Procedures

Nitrogen of enzyme was measured by the method of Johnson (4) and protein by the method of Lowry *et al.* (5). The assay of acetyl-P was carried out by the colorimetical analysis of its hydroxamate (6). Riboflavin and 6,7-dimethylribolumazine were measured as previously described (7). DPNH and TPNH were measured spectrophotometrically at 340 m μ .

RESULTS

1. Preparations of Enzymes

The following operations were carried out at 0° to 4° unless otherwise indicated.

Cl. acetobutylicum — The acetone powder of this microorganism prepared as described previously (1), was homogenized with glass powder in a small amount of 100 mM phosphate buffer (pH 5.8), and then extracted with the same phosphate buffer for one hour in an evacuated flask at room temperature. The homogenate was centrifuged. The supernatant fluid was dialyzed against 100 mM phosphate buffer (pH 5.8) for 12 hr and the protein precipitated was removed by centrifugation. Fractional precipitation with ammonium sulfate was then carried out, *i.e.*, (i) 0.3 saturation (designated as F₁), (ii) 0.3 to 0.7 saturation (F₂) and (iii) 0.7 to 1.0 saturation (F₃). After centrifugation, each fraction (F₁, F₂ and F₃) was dissolved in 100 mM phosphate buffer and dialyzed against 30 volumes of 100 mM phosphate buffer (pH 5.8) in the presence of 10 mM cysteine for 7 hr. Specific activity of each fraction was compared (Table Ia). The fraction of 0.3 to 0.7 saturation (F₂) was further treated with 1/10 volume of Amberlite IRA-400 resin.

E. coli — The dried cells prepared as described previously (7), were homogenized with glass powder in a small amount of 100 mM phosphate buffer (pH 7.0), and extracted with

TABLE I
Comparison of Enzyme Activity of Each Fraction

(a) *Cl. acetobutylicum*

Expt. no.	Fraction	Ammonium sulfate saturation	Amount of protein (N)	Flavin produced (B)	Specific activity (B/N) $\times$ 000
			mg/2 ml	μ moles/3 hr/2 ml	
1	F ₁	Up to 0.3	9.52	0.008	0.8
2	F ₂	0.3 to 0.7	6.38	0.017	2.7
3	F ₃	0.7 to 1.0	7.01	0.007	1.0

Reaction mixture contained 0.2 μ moles of 6,7-dimethylribolumazine, 10 μ moles of acetate, 2.0 μ moles of ATP, 0.2 μ moles of DPNH, 0.2 μ moles of CoA, 2.0 μ moles of MgSO₄, 200 μ moles of phosphate buffer (pH 6.6) and the enzyme of each fraction in a total volume of 2.0 ml. Incubated anaerobically for 3 hr at 37°.

(b) *E. coli neapolitanus*

Expt. no.	Fraction	Amount of protein (<i>N</i>)	Flavin produced (<i>B</i>)	Specific activity (<i>B/N</i>) × 1000
1	F ₁	6.58	0.010	1.52
2	F ₂ '	3.36	0.018	5.37
3	F ₂ ''	2.46	0.016	6.52
4	F ₃ '	2.31	0.011	4.70
5	F ₃ ''	2.03	0	—

Reaction mixture contained 0.2 μ moles of 6,7-dimethylribolumazine, 10 μ moles of acetate, equal volumes of 20 *mM* Tris-HCl and 100 *mM* phosphate buffer (pH 7.2), 0.2 ml of dialyzed extract, and the enzyme of each fraction in a total volume of 2.0 ml.

(c) *Er. ashbyii*

Expt. no.	Fractions given in Table II	Amount of protein (<i>N</i>)	Flavin produced (<i>B</i>)	Specific activity (<i>B/N</i>) × 1000
1	F ₁	0.25	0.039	156
2	F ₂	0.18	0.0074	41
3	F ₃	0.30	0.030	100
4	F ₄	0.51	0.033	65
5	F ₅	0.25	0.019	76
6	F ₆	0.27	0.041	150

Reaction mixture contained 0.2 μ moles of 6,7-dimethylribolumazine, 10 μ moles of acetate, 2.0 μ moles of ATP, 0.2 μ moles of DPNH, 0.2 μ moles of CoA, 2.0 μ moles of MgSO₄, 200 μ moles of phosphate buffer (pH 7.0), and the enzyme of each fraction as described in Table II in a total volume of 2.0 ml. Incubated for 3 hr at 37° similarly to *E. coli*.

the same buffer for 2 hr at room temperature. The homogenate was centrifuged for 40 min at 5000 rpm., and the supernatant fluid was dialyzed against 20 *mM* Tris-HCl buffer (pH 7.2) for 24 hr. The precipitated protein was removed, and the supernatant was subjected to fractional precipitation with ammonium sulfate: (i) 0.3 saturation (F₁), (ii) 0.3 to 0.7 saturation (F₂) and (iii) 0.7 to 1.0 saturation (F₃). After precipitation, each fraction (F₁, F₂ and F₃) was centrifuged and the precipitate was dissolved in 100 *mM* phosphate (pH 7.0), followed by dialysis against 20 *mM* Tris-HCl buffer (pH 7.4) in the presence of 10 *mM* glutathione. Hereupon, each dialyzed fluid was reprecipitated with ammonium sulfate: the fluid of F₁ was precipitated once more with ammonium sulfate at 0.7 saturation (F'₁). F₂ and F₃ fractions were separated further into two fractions respectively: *i.e.*, 0.5 saturation (F'₂ and F'₃) and 0.5 to 1.0 saturation (F''₂ and F''₃). Each of these fractions was dialyzed against Tris-HCl buffer as mentioned above for 8 hr. The resulting enzyme preparations were treated with 1/5 volume of Amberlite IRA-400 resin. Specific activity of each fraction was compared (Table I b).

For this alternative step, the precipitate obtained by 0.3 to 0.7 saturation (F₂) was dialyzed against 20 *mM* Tris-HCl buffer (pH 7.2) in the presence of 20 *mM* cysteine and 5 *mM* EDTA. The dialyzed solution was then adjusted to pH 6.5 with 1 *M* HCl, and was mixed with calcium phosphate gel (25 mg per 1 ml). After stirring for 30 minutes, the suspension was centrifuged and the supernatant fluid was discarded. The adsorbed gel

was washed with a small amount of 50 *mM* phosphate buffer (pH 6.5) and eluted with 100 *mM* phosphate buffer (pH 7.4). This eluate was brought to 0.6 saturation with ammonium sulfate. The precipitate was dissolved in 100 *mM* phosphate buffer (pH 6.8), and dialyzed against the buffer solution composed of equal volume of 100 *mM* phosphate and 20 *mM* Tris-HCl buffer.

Eremothecium ashbyii — 300 ml of sterile nutrient broth consisting of 1.0 % peptone, rice extracts (1 : 10), 2 % glucose, 0.1 % KH_2PO_4 , 0.05 % MgSO_4 , 2 mg % FeSO_4 , 2 mg % MnSO_4 and 0.25 % yeast extract, was adjusted to pH 6.6 with 1 *N* KOH and inoculated with *Er. ashbyii* in shaking flasks. It was kept at 30° for 48 hr in a shaking incubator. Wet mycelia were harvested and washed with 100 *mM* phosphate buffer, and were then ground mechanically. The ground mycelia were extracted with 100 *mM* phosphate buffer (pH 6.8), for 2 hr at room temperature. This fluid was centrifuged at 4000 rpm for 30 min. To the supernatant fluid, ammonium sulfate was added with stirring to make 0.3 saturation and the precipitate was discarded after centrifuging at 5000 rpm. To the fluid, ammonium sulfate was added up to 0.7 saturation. The precipitate was collected by centrifuging at 5000 rpm and dissolved in 40 ml of 20 *mM* Tris-HCl buffer solution (pH

TABLE II
Fractionation of the Enzyme Preparation of *Er. ashbyii*

7.2). This was dialyzed against 40 volumes of the same buffer containing 10 *mM* EDTA and 10 *mM* cysteine for 15 hr. To the dialyzed solution 2 ml of 1% protamine solution was added with stirring. After 5 min, the solution was centrifuged and the precipitate was discarded. To the supernatant fluid, 5 ml of 1% protamine was added and centrifuged. The supernatant fluid was treated as shown in Table II. The gummy precipitate was washed twice with a total volume of 15 ml of cold water by centrifuging. The precipitate was eluted with 30 ml of *M*/15 phosphate buffer (pH 7.3), and then dissolved in 30 ml of 250 *mM* phosphate buffer (pH 7.5). To this eluate, ammonium sulfate was added up to 0.7 saturation. By this treatment, a large amount of riboflavin present in the enzyme solution was almost removed. The precipitate was centrifuged down and dissolved in 15 ml of Tris-HCl buffer (pH 7.2). This was dialyzed for 8 hr in the same manner as mentioned above. To the dialyzed solution, calcium phosphate gel (approximately 15 mg dry weight per ml) was added. After 5 min, the gel was centrifuged down and the supernatant fluid was discarded. The gel was then washed with a small amount of 50 *mM* phosphate buffer (pH 6.4). The gel was eluted with 100 *mM* phosphate buffer, (pH 7.4). This eluate was brought to 0.7 saturation with ammonium sulfate. The precipitate was dissolved in 100 *mM* phosphate buffer (pH 6.8), and dialyzed against a buffer solution composed of equal volumes of 100 *mM* phosphate and 20 *mM* Tris-HCl buffer. This purification is a modification of Berg's method (8). For alternative steps, the eluate of calcium phosphate gel was treated with Norit, and ammonium sulfate was added up to 0.7 saturation as mentioned above.

Beef Liver — Fresh beef liver obtained from the slaughter-house, was carefully packed in ice, and the following step was accomplished within 1 to 2 hr. The liver was cooled in crushed ice and finely minced by a mincing machine. The mince was homogenized in a blender with 1.5 volumes of 100 *mM* phosphate buffer. From the homogenate, acetone powder was prepared in the same manner as described previously (1). The acetone powder of liver was homogenized mechanically with the same buffer (pH 6.8), and the extract was centrifuged. The supernatant fluid was dialyzed against 50 volumes of buffer solution composing of equal volumes of 100 *mM* phosphate and 20 *mM* Tris-HCl buffer (pH 7.2), in the presence of 20 *mM* cysteine and 10 *mM* EDTA. This crude cell-free extract was employed for fractional precipitation. The fraction of 0.3 to 0.7 saturation with ammonium sulfate was dissolved in 100 *mM* phosphate buffer, dialyzed against the same buffer for 12 hr, followed by reprecipitation by ammonium sulfate saturation. After dialysis against 20 *mM* Tris-HCl buffer (pH 7.2), it was treated with 1/2 volume of Dowex 1-chloride form.

Lactobacillus plantarum and *Lactobacillus casei* — Both bacteria were grown at 37° for 40 hr in a culture medium composing of malt extract containing 2% glucose, 0.5% yeast extract and 2.0% sterilized CaCO₃, which had been adjusted to pH 7.0 with 1 *N* KOH. The cells were harvested by the Sharples centrifuge and washed with distilled water. The harvested cells were dried with an electric fan at room temperature. The dried cells were suspended in a small amount of 100 *mM* phosphate buffer (pH 7.0), ground mechanically, and then put into equal volumes of 100 *mM* phosphate buffer and 20 *mM* Tris-HCl buffer (pH 7.2). It was centrifuged at 5000 rpm for 40 min and the supernatants were used as the crude extract in both cases of bacteria.

Neurospora crassa — The cells were grown aerobically on a shaker at 30° for 48 hr in a culture medium consisting of 2 g of glucose, 50 mg of K₂HPO₄, 50 mg of KH₂PO₄, 20 mg of MgSO₄, 1 mg of FeSO₄, 1 mg of NaCl, 500 mg of peptone, 500 mg of sodium tartarate, 100 mg of sodium nitrate, and 100 ml of distilled water, which had been adjusted to pH 6.4 with 1 *N* KOH. The wet mycelia were harvested, washed with 100 *mM* phosphate buffer and ground mechanically. The ground mycelia were extracted with 100 *mM* phos-

phate buffer (pH 7.0), for 1 hr at room temperature. This fluid was centrifuged at 4000 rpm for 30 min. To the light brown supernatant fluid, ammonium sulfate was added with stirring, and fraction of 30 to 70 per cent saturation was collected. The precipitate was collected by centrifuging and dissolved in 20 mM Tris-HCl buffer (pH 7.2), followed by dialysis against the same buffer in the presence of 5 mM EDTA and 10 mM cysteine for 18 hr. The resulting enzyme preparation was treated with 1/5 volumes of Dowex-chloride form resin.

2. Properties of Enzyme

After dialysis of the enzyme solutions of *E. coli*, beef liver and *Er. ashbyii*, more active preparations were obtained with Tris-HCl buffer than with phosphate buffer. Moreover, the addition of glutathione or cysteine was necessary to secure the highest activity in the course of dialysis. In the experiments with the enzyme preparations of *Cl. acetobutylicum* and *E. coli*, it was found that the enzyme system was, as shown in Table III, completely inhibited in the presence of 0.01 M *p*-chloromercuribenzoate and inhibited by 43% with

TABLE III
Effect of Some Compounds on Enzyme Action of *E. coli neapolitanus*

Exp. no.	Addition	Percentage of maximal activity
1	0.01 M <i>p</i> -chloromercuribenzoate	0
2	" " plus 0.01 M GSH	33
3	" iodoacetate	57
4	" " plus 0.01 M GSH	72
5	" fluoroacetate	100
6	" fluoroacetamide	59
7	" KCN	100
8	" NaF	100
9	" arsenate	69
10	" arsenite	52

The reaction system contained 20 μ moles of phosphate buffer (pH 7.0) except for Nos. 9 and 10, partially purified enzyme treated with Ca gel (370 μ g nitrogen), 0.2 μ moles of 6,7-dimethylribolumazine, 10 μ moles of acetyl-P and 0.2 μ moles of DPNH in a final volume of 2.0 ml. Incubation at 37°.

0.01 M monoiodoacetate, but the inhibition was partially diminished by the addition of 0.01 M glutathione. The inhibitory effect of several metallic ions has been tested with the enzyme preparation of *E. coli*. As is summarized in Table IV, the metals tested here, could be classified into three groups according to their effects as follows:

(i) Ag, Hg, Cu, Zn and Fe caused complete or definite inhibition at the concentration of $10^{-2}M$.

(ii) Co produced 50 per cent inhibition, and Mn 33 percent, whereas inhibition by Ni was rather slight.

(iii) Other ions such as Mo, Li and Mg revealed no inhibition at this concentration. It has been observed with *Cl. acetobutylicum* (9) that iron produced an inhibitory action to riboflavin formation, but without effect on the growth or fermentation of the bacterium. Katagiri and Imai have reported (10) that

TABLE IV
Effect of Some Metal Ions on Enzyme Action of *E. coli neapolitanus*

Expt. no.	Addition of metal salt (20 μ moles)	Percentage of maximal activity
1	AgNO ₃	0
2	HgCl ₂	0
3	HgCl ₂ plus 0.05 M cysteine	8
4	CuSO ₄	0
5	ZnSO ₄	16
6	FeSO ₄	24
7	CoSO ₄	50
8	MnSO ₄	67
9	NiSO ₄	79
10	MgSO ₄	100
11	Na ₂ MoO ₄	100
12	Li ₂ SO ₄	100

Reaction system contained 20 μ moles of metal salt, 0.2 μ moles of 6,7-dimethyl-riboflumazine, 10 μ moles of acetylphosphate, 0.2 μ moles of DPNH, 20 μ moles of phosphate buffer (pH 7.0) and partially purified enzyme with 370 μ g nitrogen in a final volume of 2.0 ml. Incubation was made for 3 hr at 37°.

several metals including Fe, Co and Ni inhibited riboflavin formation with this kind of microorganism. These results are in agreement with the findings observed above. Enzymatic activities of the preparations were highly maintained when dialyzed in the presence of EDTA. The activation caused

FIG. 1 Progress Curve of *E. coli neapolitanus*

Reaction mixture contained 0.2 μ moles of 6,7-dimethylriboflumazine, 10 μ moles of acetyl-P, 0.2 μ moles of DPNH, 20 μ moles of phosphate buffer (pH 7.0) and 240 μ g of nitrogen of partially purified enzyme treated with Ca gel in a total volume of 2.0 ml. Each point represents a separate incubation for 3 hr at 37°.

FIG. 2 pH Curve of *E. coli neapolitanus*

Incubation at 37° for 3 hr. Reaction system contained 0.2 μ moles of 6,7-dimethylriboflumazine, 10 μ moles of acetylphosphate, 0.2 μ moles of DPNH, 200 μ moles of phosphate buffer and 240 μ g of partially purified enzyme treated with Ca gel in a total volume of 3.0 ml. Each point represents a separate incubation.

by EDTA is possibly due to the removal of heavy metals. The enzyme preparations of *E. coli* and *Er. ashbyii* were stored at -10° , under which conditions it was found to be stable at least for three weeks.

The progress curve shown in Fig. 1 was obtained by a partially purified preparation of *E. coli*. It will be seen that the initial period of reaction is a latent phase, followed by a proportional phase from the time of 2 hours at 37° . The maximum activity was observed at pH 7.0 in phosphate buffer using the same enzyme (see Fig. 2). Experiments were not carried out on an alkaline side, since 6,7-dimethylribolumazine was very sensitive to alkali. Inactivation of the enzyme caused by heating, was found to be about 45 per cent when heated for 5 min at 50° and complete when heated for 5 min at 65° .

The relationship between enzyme concentration and reaction rate was shown in Fig. 3. Fig. 4 shows the effect of the concentration of 6,7-dimethylribolumazine on the rate of riboflavin formation. Maximum activity was found at about 1 mM of 6,7-dimethylribolumazine. From the data in Fig. 4, the concentration of 6,7-dimethylribolumazine giving half maximum activity was found to be about 0.1 mM.

Various kinds of compounds were tested for their effects on the activity of riboflavin synthesis by the preparation of *E. coli* (Table III). The addition of 0.01 M fluoroacetate did not show any measurable inhibition in the presence of acetate, but 0.01 M monofluoroacetamide produced 41 per cent inhibition. It was found that KCN and NaF did not exert any inhibition at the concentration of 0.01 M. Sodium arsenite and sodium arsenate were found to inhibit

FIG. 3 Enzyme Concentration and Reaction Rate of *E. coli neapolitanus*

Assay procedure was the same as given in Fig. 1 except for the concentration of the enzyme.

FIG. 4 6,7-Dimethylribolumazine Concentration and Riboflavin Production of *E. coli neapolitanus*

Assay procedure was the same as given in Fig. 1 except for the concentration of 6,7-dimethylribolumazine

48 and 31 per cent, respectively. The inhibition caused by arsenate may be due to arsenolysis of acetylphosphate, which is an important donor of riboflavin synthesis.

3. Distribution

The enzymes involved in riboflavin synthesis have been found in various kinds of microorganisms and animal tissues (Table V). Further studies would

TABLE V
Activity of Enzyme Preparations

Exp. no.	Origin of enzyme	Amount of protein (A)	Flavin produced (B)	Specific activity (B/A) $\times$ 1000
		mg/2 ml	μ moles/2 ml	
1	<i>Er. ashbyii</i> (F ₁ in Table II)	0.21	0.041	195
2	<i>E. coli</i> (Ca-gel treated enzyme)	1.29	0.042	32.6
3	Beef liver	2.47	0.032	13.0
4	<i>Neurospora crassa</i>	1.82	0.017	9.35
5	<i>Cl. acetobutylicum</i>	7.22	0.019	2.76
6	<i>L. plantarum</i>	—	+	—
7	<i>L. casei</i>	—	—	—

Reaction mixture contained 200 μ moles of phosphate buffer (pH 7.2), 40 μ moles of Tris-HCl buffer (pH 7.2), 0.2 μ moles of 6,7-dimethylribolumazine, 10 μ moles of acetate, 0.2 μ moles of DPNH, 0.2 μ moles of CoA, 2.0 μ moles of ATP, 2.0 μ moles of MgSO₄, and enzyme preparations described in the text in a total volume of 2.0 ml. It was incubated at 37° for 3 hr.

be necessary to ascertain the negative result obtained with the enzyme preparation of *Lactobacillus casei*, but it is clear that the enzyme is widely distributed in nature. It is very interesting that the enzymes from both beef liver and *E. coli* revealed considerable activities for conversion of 6,7-dimethylribolumazine into riboflavin, taking into consideration of the fact that they have scarcely any ability of riboflavin formation in the normal condition. Among a number of organisms tested, *Er. ashbyii* has shown the maximal activity. From these experiments, it is concluded that 6,7-dimethylribolumazine is a direct precursor of riboflavin synthesis. The reason why *Cl. acetobutylicum* represented less activity than *E. coli* and beef liver, will be discussed later.

4. Kinds of Cofactors

It will be seen in Table VI that the activity of the enzyme preparation capable of riboflavin synthesis from 6,7-dimethylribolumazine and acetate fell markedly to almost negligible after prolonged dialysis and passage through an anion exchange resin, indicating the removal of the cofactors necessary for the progress of the enzymatic reaction. Since the reaction system requires acetate as a 4-C-donor of 6,7-dimethylribolumazine, acetate must be activated in some way prior to the participation in this reaction system. Table VI shows that the enzyme of *E. coli* for riboflavin synthesis is much stimulated by the addition of ATP and DPNH. A great activity as observed in the absence

of CoA or acetate, may possibly be due to the impurity contained in the enzyme preparation and the contamination of acetate in the ATP preparation. This enzyme system seems to be much more sensitive to acetate than 6,7-dimethylribolumazine, as will be reported in the following paper. Riboflavin synthesis from 6,7-dimethylribolumazine with the enzyme of *Cl. acetobutylicum* was much decreased in the absence of DPNH, ATP and CoA (Table VII), but DPN failed to stimulate in both preparations of *E. coli* and *Cl. acetobutylicum*. As for the stimulative effect of ATP, the enzyme of *E. coli* was promoted

TABLE VI
*Enzymatic Conversion of 6,7-Dimethylribolumazine to Riboflavin Using
the Preparation of E. coli neapolitanus*

Expt. no.	Reaction system	Riboflavin produced 10 ⁻³ mM
1	All	2.1
	No ATP	0.51
	No CoA	1.5
	No DPNH	0.43
	No Mg	2.2
	No acetate	1.2
	No cofactors	0.31
	Pyruvate, TPP, Mg and DPN	0.44
	All plus α -ketoglutarate	2.5
2	All	1.7
	Acetyl-P in place of acetate and ATP	1.8
	TPNH in place of DPNH	1.6
	All plus 10 mM EDTA	1.2
	Acetyl-P, CoA, DPNH, Mg and 10 mM EDTA	1.7

Reaction system: All, composed of all the substances, *i.e.*, 0.2 μ moles of 6,7-dimethylribolumazine, 10 μ moles of acetate, 2.0 μ moles of ATP, 0.2 μ moles of DPNH, 0.2 μ moles of CoA, 200 μ moles of phosphate buffer (pH 7.0) and 2.0 μ moles of MgSO₄. Other substances used were as follows: 10 μ moles of acetyl-P, 0.2 μ moles of TPNH, 2.0 μ moles of TPP, 10 μ moles of pyruvate and 5 μ moles of α -ketoglutarate. The final volume was adjusted in each case to 2.0 ml. The enzyme treated with calcium phosphate gel was used. Reaction mixture contained 370 μ g nitrogen of enzyme in the experiment 1 and 340 μ g in the experiment 2. The reaction was carried out at 37° for 3 hr.

by about 32 per cent with the increase of phosphate buffer concentration from 50 to 100 mM. TPNH showed less stimulative effect than DPNH at the same concentration. In the presence of acetaldehyde, DPNH, TPP and Mg, the activity of the enzyme preparation of *Cl. acetobutylicum* was low. It is not likely that acetaldehyde forms acetoin by the action of carboligase which has been observed in yeast, wheat germ and in some microorganisms, and that riboflavin synthesis takes place from acetoin and 6,7-dimethylribolumazine. If the pathway of acetate activation by ATP through the action of acetokinase (11), followed by the transfer of the acetyl group to CoA through the action of phosphotransacetylase (12), the role of acetate and ATP in the riboflavin biosynthesis from 6,7-dimethylribolumazine may be replaced by acetyl phosphate. The addition of acetyl phosphate prepared chemically by the method of Avison (3) could really replace ATP and acetate (see Table VI and VII).

TABLE VII
Enzymatic Conversion of 6,7-Dimethylribolumazine to Riboflavin Using
the Preparation of *Cl. acetolutylicum*

Expt. no.	Reaction system	Riboflavin produced 10 ⁻² mM
1	All	0.87
2	No ATP	0.28
3	No CoA	0.32
4	No DPNH	0.62
5	No Mg	0.94
6	No acetate	0.60
7	No 6,7-dimethylribolumazine	0
8	Acetyl-P in place of acetate and ATP	0.94
9	TPNH in place of DPNH	0.68
10	TPP, DPNH, Mg, ATP, CoA and acetaldehyde	0.83
11	TPP, DPNH, Mg and acetaldehyde	0.18
12	All plus 10 mM EDTA	0.52

Reaction system: All, composed of all the substances, *i.e.*, 0.2 μ moles of 6,7-dimethylribolumazine, 10 μ moles of acetate, 2.0 μ moles of ATP, 0.2 μ moles of DPNH, 0.2 μ moles of CoA, 2.0 μ moles of MgSO₄ and 200 μ moles of phosphate buffer (pH 6.6). Other substances used were as follows: 10 μ moles of acetyl-P, 0.2 μ moles of TPNH, 2.0 μ moles of TPP, 10 μ moles of acetaldehyde and 1,160 μ g nitrogen of enzyme treated with Amberlite IRA-400 resin as described in the text. Incubated anaerobically for 3 hr at 37°.

The activity of acetokinase in both preparations of *Cl. acetobutylicum* and *E. coli* was tested according to the assay procedure of Rose (11). The high activity of this enzyme was ascertained by a paper chromatogram of acetyl-P and by the determination of acethydroxamate. The supplement of Mg to both preparations of *Cl. acetobutylicum* and *E. coli* was proved to be inactive in this enzyme system. But the addition of EDTA at the concentration of 10 mM in the reaction mixture inhibited riboflavin synthesis, when EDTA and the enzyme were preincubated before adding the substrates. The inhibitory

TABLE VIII
Enzymatic Conversion of 6,7-Dimethylribolumazine into Riboflavin Using
the Preparation of *Er. ashbyii*

Expt. no.	Reaction system	Riboflavin produced 10 ⁻² mM
1	All	2.0
2	No acetate	0.67
3	No ATP	1.0
4	No CoA	1.6
5	No Mg	1.9
6	Diacetyl in place of acetate	0.76

Reaction system: All, composed of all the substances, *i.e.*, 0.2 μ moles of 6,7-dimethylribolumazine, 10 μ moles of acetate, 2.0 μ moles of ATP, 0.2 μ moles of DPNH, 0.2 μ moles of CoA, 200 μ moles of phosphate buffer and 2.0 μ moles of MgSO₄. 10 μ moles of diacetyl was used in the experiment No. 6. The total volume was adjusted in each case to 2.0 ml. Enzyme of fraction F₁ contained 36 μ g nitrogen. The reaction was carried out at 37° for 3 hr.

effect of EDTA was not seen, when acetyl-P was added in place of acetate and ATP (Table VI). Results obtained with the preparation of *Er. ashbyii* are summarized in Table VIII, which excludes the role of diacetyl as a 4-C-donor, as already reported in the previous paper (7). For a satisfactory reactivation of this enzyme, the simultaneous addition of all cofactors was found necessary; *i.e.*, ATP, DPNH, CoA and Mg.

DISCUSSION

Among a number of organisms tested, *Er. ashbyii* revealed the maximum activity of riboflavin synthesis from 6,7-dimethylribolumazine as given in Table V. From these results, this lumazine compound was proved to be a direct intermediate of riboflavin synthesis. Although *Cl. acetobutylicum* produced a comparatively large amount of riboflavin and a moderate amount of 6,7-dimethylribolumazine in the culture filtrate as already described (13), the enzymatic activity of riboflavin formation from 6,7-dimethylribolumazine by this microorganism was at least comparable with both *E. coli* and beef liver. The content of 6,7-dimethylribolumazine in the culture filtrate of *Er. ashbyii* was 1/50 that of riboflavin in the 84-hr culture (14). On the other hand, the ratio of 6,7-dimethylribolumazine to riboflavin in *Cl. acetobutylicum* was 1/7 in the 72-hr culture in the synthetic medium. The accumulation of such a great amount of 6,7-dimethylribolumazine in the culture filtrate of the latter suggests that the enzymatic activity of riboflavin synthesis from this lumazine compound is very low as compared with *Er. ashbyii*. Another reason may be due to the loss of activity in the course of treatment, because this enzyme preparation is much more labile than other preparations from various sources. The most striking finding is that such a high activity is observed in a liver preparation in the presence of acetate, 6,7-dimethylribolumazine and various cofactors as shown in Table V. Although animal tissues have been supposed to have no ability for riboflavin production, the production of the vitamin by a soluble enzyme extracted from beef liver suggests that this enzyme is widely distributed and is of non-specific nature. It is most likely that the enzymes catalyzing the conversion of 6,7-dimethylribolumazine into riboflavin are widely distributed in nature, since riboflavin is produced more or less by most microorganisms and plants. A homofermenter, *Lactobacillus plantarum*, which produces a small amount of riboflavin, FMN, FAD and a trace of 6,7-dimethylribolumazine in the cells, reveals a small activity of enzymatic conversion of 6,7-dimethylribolumazine into riboflavin, whereas negative results were obtained with the cell-free extracts of *Lactobacillus casei*. Further experimental evidence will be necessary to conclude the absence of this enzyme in this microorganism.

ATP and DPNH were found to activate prominently this enzyme system in the case of *Cl. acetobutylicum* and *E. coli*, while DPNH was not effective in the enzyme preparation of *Er. ashbyii*. In *Er. ashbyii*, DPN might be bound to its enzyme preparation in a sufficient amount for the maximal activity. Further purification is therefore necessary.

A satisfactory explanation for the activity of TPNH in the partially purified enzymes of *E. coli* and *Cl. acetobutylicum* has not yet been established, but it seems to be certain that these enzymes perhaps require DPNH as an electron donor.

Juni (15) has reported that the formation of acetoin by bacteria takes place via α -acetolactic acid from pyruvic acid. In the case of *E. coli neapolitanus*, the present authors could not find the production of acetoin or 2,3-buteneglycol in its culture filtrate when glucose was used as a carbon source. In the presence of pyruvate, DPN and TPP, the enzymatic activity was minimal as shown in Table VI. Moreover, diacetyl was inactive as a 4-C-donor. Acetaldehyde, on the other hand, was not utilized directly for the formation of riboflavin from 6,7-dimethylribolumazine (1). In the enzyme preparations of *Cl. acetobutylicum* and *E. coli*, it is most likely that the activation of acetate is initiated by phosphorylation, followed by the transfer of acetyl group to CoA.

Although the precise mechanisms of A ring formation in riboflavin synthesis must await further investigation, it is suggested that acetylation and reduction take place at first, followed by ring closure.

The mechanism of riboflavin synthesis from 6,7-dimethylribolumazine seems to be the same, regardless of the preparations obtained from various kinds of organisms. When the reaction mixtures after incubation were subjected to paper chromatography according to Crammer, green fluorescent compounds having R_F values of 0.30 and 0.14 respectively after development with a butanol-pyridine-water system (6:4:3) were detected, apart from the spots of a violet fluorescent compound (R_F 0.17) and of riboflavin. The R_F values and nature of fluorescence of these green compounds were found to correspond with G_1 and G_2 Compounds detected in the culture filtrate of *Cl. acetobutylicum* (13).

SUMMARY

1. The enzyme catalyzing the conversion of 8-*N*-ribityl-6,7-dimethylumazine to riboflavin was found in *Er. ashbyii*, beef liver, *L. plantarum*, *Neurospora crassa*, as well as in *E. coli* and *Cl. acetobutylicum*, in which its existence has already been reported.

2. The enzyme is widely distributed in nature, and among a number of organisms tested, the activity of *Er. ashbyii* was the highest.

3. The activity of the partially purified enzyme of *E. coli neapolitanus* was inhibited by sulfhydryl reagents such as *p*-chloromercuribenzoate and monoiodoacetate. A remarkable inhibition was also produced by several heavy metal ions at the concentration of 10^{-2} *M*, i.e., Ag, Hg, Cu, Zn and Fe. KCN, NaF and monofluoroacetate did not exert any inhibition at 0.01 *M*.

4. The effect of the concentrations of 6,7-dimethylribolumazine and of the enzyme as well as that of pH on the rate of riboflavin synthesis were examined.

5. For full activation of this enzyme, it requires the addition of such cofac-

tors as ATP, DPNH and CoA. Acetyl phosphate is found to be replaceable for acetate and ATP, when the enzyme preparations of *Cl. acetobutylicum* and *E. coli*. were used.

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