STUDIES ON THE INTERMEDIATES IN THE BIO-SYNTHETIC PATHWAY OF RIBOFLAVIN

I. IDENTIFICATION OF A GREEN FLUORESCENT COMPOUND, COMPOUND G₁, ACCUMULATED IN NON-GROWING CELLS OF *EREMOTHECIUM ASHBYII* BY THE ADDITION OF DIMERIC DIACETYL¹

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Summary Effects of sugars and carbonyl compounds (1%) on riboflavin formation were examined with non-growing cells of *Eremothecium ashbyii*. During non-growing cell incubation for 18 hr, the addition of glucose markedly stimulated the formation of riboflavin, but the addition of pentoses (ribose, xylose, arabinose), ribitol and acetoin indicated no effect on the production. The supplementation of pyruvate, acetate, diacetyl, glyoxal and acetoacetate, on the other hand, noticeably inhibited the formation of riboflavin.

The effects of diacetyl monomer, dimer and trimer on the formation of riboflavin were further examined. Dimeric diacetyl (0.5%) among the diacetyl derivatives caused the accumulation of two green fluorescent compounds inside and outside of the cells during the 18 hr incubation. The fraction (Compound G₁), which was first eluted in Dowex 50W×4 column chromatography, was accumulated in large amounts indicating an inverse relation to riboflavin formation.

Compound G₁ was isolated from the mycelia and highly purified through many purification procedures. The structure of compound G₁ was studied by paper chromatographic comparison, by determination of ultraviolet, emission, excitation and infrared spectra, by following the change of the maximum fluorescence of the reaction mixture containing the isolated compound in the presence of *p*-quinone and by examining the possibility of it as a substrate for riboflavin synthetase. The results obtained indicated that the isolated compound G₁ is 6,7-dimethyl-8-(1'-D-ribityl)

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lumazine.

We speculated that dimeric diacetyl or its derivative becomes a significant inhibitor for the riboflavin synthetase reaction to result in the accumulation of 6,7-dimethyl-8-ribityllumazine although the true inhibitor remains to be solved in future.

Many studies were done using glyoxal (1) and diacetyl (2-6) as trapping agents for the expected intermediates in the riboflavin biosynthetic pathway. The authors isolated a possible intermediate using glyoxal from a high flavinogenic mold, *Eremothecium ashbyii* (7) and an adenine-riboflavin doubleless mutant of *Bacillus subtilis* (8). The intermediate was proved to be 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine after crystallization through high purification by means of various column chromatographic analyses. Furthermore, the behavior of the accumulation of the pyrimidine indicated that the pyrimidine is an immediate intermediate to 6,7-dimethyl-8-ribityllumazine in the biosynthetic pathway of riboflavin. In the present paper, isolation of possible intermediates were further attempted with dimeric diacetyl, a trapping agent, but not with diacetyl in a high flavinogenic strain of *E. ashbyii*.

MATERIALS AND METHODS

Materials. Diacetyl was purchased from Nakarai Chemicals, Ltd. Dimeric diacetyl was prepared by self-condensation of diacetyl in an alkaline solution with 1 N KOH (9). Dimeric diacetyl used in the experiment shown in Table 3 was purified by distillation under reduced pressure. Trimeric diacetyl was obtained by keeping diacetyl with Amberlite CG400 (OH⁻) at room temperature for 4-5 days (10). All other chemicals were of the best quality available.

Organism. *Eremothecium ashbyii* Guilliermonde IFO 0944 was obtained from the Institute for Fermentation, Osaka, was transplanted periodically on agar slants containing 1% glucose, 1% peptone, 0.1% yeast extract and 1.2% agar (pH 6.5).

Non-growing cell incubation. A mycelial fragment from a 7-day agar slant was transferred to the precultural medium containing the same nutrients as those in the agar slant except for agar and cultured at 27°C for 24 hr. Then, 5 ml of the culture was transferred to the basal medium (500 ml) consisting of 2% glucose, 1% peptone, 0.3% yeast extract, 0.2% KH₂PO₄, 0.1% NaCl, 0.01% MgSO₄·7H₂O and 1.8% Tween 80 (pH 5.5), followed by cultivation on a rotary shaker (220 rpm) at 27°C for 24 hr. Mycelia were harvested by filtration and washed well with distilled water. The washed mycelia were starved by mild shaking for 8 hr in 0.1 M phosphate buffer (pH 6.8) containing 0.5% glucose. After filtration and washing, the starved cells were resuspended in the same buffer containing test compounds and submitted to vacuum infiltration treatment (11). The cell suspensions were incubated with moderate shaking in the dark at 27°C for 18 hr.

The incubated mixture was separated into cells and incubation fluid by filtration. The cells were suspended in distilled water at a ratio of 5 ml/g wet mycelia and heated in darkness at 80°C for 10 min. The extract obtained by centrifugation was used for the determination of absorbance at 450 nm with the fluid mentioned above and for isolation of accumulated compounds in the presence of dimeric diacetyl. The flavin contents in the mycelia and in the incubation fluid were separately calculated from the absorbance at 450 nm using the millimolar absorption 11.2. Thus, the flavin contents correspond to the sum of the respective amounts of riboflavin, FMN and FAD. Then, the amounts of flavins in the mycelia and those in the incubation fluid were summed up and expressed as total flavins in weights per the initial wet mycelia ($\mu\text{g/g}$) used for the non-growing cell incubation.

Isolation and purification of green fluorescent compound (G_1) accumulated. The mycelia which have been incubated for 18 hr with 0.5% dimeric diacetyl were extracted by heating them at 80°C for 15 min. The slurry was filtered. The filtrate (100 ml) was applied to a Dowex 50W $\times$ 4 (H^+ , 200–400 mesh) column (2.2 $\times$ 30 cm) and eluted with distilled water at a flow rate of 21 ml/5 min. Two green fluorescent compounds were isolated on the chromatogram as seen in Fig. 1. The first green fluorescent compound eluted was referred to as compound G_2 and

Fig. 1. Column chromatography of mycelial extract on Dowex 50W $\times$ 4. The non-growing mycelia of *Eremothecium ashbyii* fed with 0.5% dimeric diacetyl were heated in distilled water at 80°C for 15 min and extracted. The extract (100 ml) was applied to a column (2.2 $\times$ 30 cm) on Dowex 50W $\times$ 4 (H^+ , 200–400 mesh). Chromatography was carried out with distilled water at a flow rate of 21 ml/5 min. Absorbances of eluate were automatically recorded simultaneously at 260, 280 and 440 nm with a Hitachi 034 UV-VIS effluent monitor. FAD, flavin adenine dinucleotide; V, 6-methyl-7-hydroxy-8-ribityllumazine; G_1 , G_2 , green fluorescent compounds; B_2 , riboflavin.

the second as compound G_1 . The accumulated amounts of the latter were much larger than those of the former. Then, the compound G_1 fractions were collected and loaded on to a Florisil (magnesium silicate) column (2.2×20 cm) which was preliminarily equilibrated with 5% acetic acid. The green fluorescent fraction eluted with 5% pyridine was dried under reduced pressure. The dried matter was dissolved in a minimum volume of water and run through a column (1.5×90 cm) on Sephadex G-10. Chromatography was carried out with 0.05 M phosphate buffer (pH 7.0). The obtained green fluorescent fluid was applied again to a Florisil column (2.2×20 cm) and eluted in the same manner described above. The eluate having a green fluorescence was collected and dried *in vacuo*. The residue was submitted to identification of compound G_1 as follows.

Determination of compound G_1 accumulated. The mycelia incubated in the phosphate buffer containing various concentrations of dimeric diacetyl for 18 hr were extracted in the way described in *Isolation and purification*. Then, an appropriate volume of the incubation fluid and the extract was submitted to paper chromatography with a solvent system of butanol: pyridine: H_2O (6: 4: 3). Chromatography was done overnight by the ascending method at room temperature. The paper was completely dried in the dark and the fluorescent spots were located under ultraviolet light. The green fluorescent band attributable to compound G_1 on the paper was cut and the fluorescent compound was extracted by heating it with a constant volume of distilled water on a water bath at $80^\circ C$ for 10 min. The amounts of the compound were fluorometrically determined by comparing them with those of a reference compound, 6,7-dimethyl-8-ribityllumazine and expressed as $\mu g/g$ wet mycelia in the sum of the amounts from the incubation medium and the amounts from the extract.

Identification of the purified compound G_1 . For identification of compound G_1 , paper chromatography was done by the ascending method at room temperature overnight with the solvent systems described in the footnotes of Tables 1 and 2. Ultraviolet and visible absorption spectra of the purified compound G_1 were followed over a wavelength of 210–460 nm in acid or alkaline solution with a Shimadzu multipurpose recording spectrophotometer MPS-50L. Excitation and emission spectra of the isolated compound G_1 and the produced compound due to *p*-quinone were measured in 0.05 M phosphate buffer (pH 7.0) by a Hitachi fluorescence spectrophotometer MPF-2A. The infrared spectra of compound G_1 and an authentic compound were determined on KBr pellets with a Hitachi infrared spectrophotometer EPI-G3.

Preparation of riboflavin synthetase. The enzyme, riboflavin synthetase, used to verify the conversion of compound G_1 to riboflavin, was prepared by following procedures.

Mycelial pad (100 g wet weight) of *E. ashbyii* obtained after 2-days' cultivation was ground for 10 min in a mortar with 150 g of alumina (bacterial grade). The slurry was homogenized in 200 ml of 0.1 M potassium phosphate buffer (pH 6.5)

containing 10 mM Na_2SO_3 , 10 mM mercaptoethanol and 5 mM EDTA (buffer A). The homogenate was centrifuged at 8,000 rpm for 15 min. The supernatant was adjusted to pH 5.5 with 3 M acetic acid, and allowed to stand overnight. The resulting precipitate was discarded by centrifugation. The pH of the supernatant was adjusted to 6.5 with 1 N NaOH. Into the solution, solid ammonium sulfate was added to give 38% saturation with stirring for 2 hr. The precipitate was removed by centrifugation. Ammonium sulfate was added again into the supernatant to 58% saturation. After centrifugation, the obtained precipitate was dissolved in buffer A to give a protein concentration of about 15 mg/ml buffer A. Mercaptoethanol was added to the above mixture at 0.2 M in a final concentration. The solution was kept in a stocker (-20°C) and allowed to settle overnight in a frozen state. Then, the material was thawed in a water bath at 10°C . The resulting suspension was centrifuged and the supernatant obtained was retained. The precipitate was suspended in buffer A and homogenized. After centrifugation, the supernatant was combined with the fluid obtained after the first centrifugation. The combined supernatant was subjected to ammonium sulfate treatment (38–58% saturation) procedure described above. Operations in all steps were undertaken in the cold ($0-4^\circ\text{C}$) unless otherwise stated.

The enzyme reaction was allowed to proceed in darkness at 37°C for the indicated times, followed by a periodical measuring of the changes of absorbances in the 340–500 nm visible region in a cuvet (final volume, 1 ml) containing certain amounts of the purified compound G_1 , the enzyme protein 0.18 mg, 5 mM Na_2SO_3 , 2.5 mM mercaptoethanol and 2.5 mM EDTA in 50 mM potassium phosphate buffer (pH 6.5).

RESULTS

1. *Effects of sugars and carbonyl compounds on riboflavin formation of non-growing cells of E. ashbyii*

The cell suspensions containing various sugars and carbonyl compounds at 1% were submitted to the non-growing cell incubation for 18 hr. The yields of riboflavin are given in Fig. 2. The results indicate that ribose, ribitol, xylose, arabinose and acetoin provided values almost similar to control, although glucose exerted a noticeable stimulatory effect. While, pyruvate, acetate, diacetyl, glyoxal and acetoacetate, especially acetate and diacetyl, preferably caused the rigid inhibition of riboflavin formation. It is to be noted that diacetyl markedly restricted riboflavin formation whereas acetoin had no effect although both compounds are near metabolites in the acetoin biosynthetic pathway.

2. *Effects of diacetyl derivatives, monomer, dimer and trimer on riboflavin formation of the non-growing cells of E. ashbyii*

The mono-, di- and tri-mer of diacetyl were added to the incubation medium at 0–1% concentration and the incubation was done for 18 hr after vacuum in-

Fig. 2. Effects of sugars and carbonyl compounds on riboflavin formation. Sugars and carbonyl compounds indicated in the figure were added at 1% to the non-growing cell medium (pH 6.8) and was treated with vacuum infiltration. After the incubation at 27°C for 18 hr, the yields of riboflavin were measured as described in MATERIALS AND METHODS.

filtration. The resulting riboflavin content is shown in Fig. 3. The addition of diacetyl monomer caused a marked inhibition of the formation up to 0.2% concentration, attaining the plateau region above the concentration. The addition of diacetyl dimer at 0.1% stimulated the formation and inhibited the formation with increasing concentration above 0.2%. While, the supplementation of a diacetyl trimer exerted a mild inhibitory effect with its increasing concentrations. The incubation fluid and the aqueous extract from the mycelia under experimental conditions were subjected to paper chromatographic analysis with a solvent, butanol: pyridine: H₂O (6: 4: 3). The results indicated that a small amount of an unknown blue violet fluorescent spot (R_f 0.25) was detected in the additions of diacetyl monomer and trimer under ultraviolet light. Whereas, in the addition of diacetyl dimer, the accumulation of a green fluorescent compound (R_f 0.20) was observed in a large amount.

3. Relation between the production of riboflavin and the accumulation of a green fluorescent compound in the presence of dimeric diacetyl

Determination of the amounts of riboflavin formed and compound G₁ accumulated was carried out after an 18 hr incubation in the presence of dimeric diacetyl at various concentrations. As seen in Fig. 4, the behavior of riboflavin formation indicated a similar attitude compared to that in Fig. 3. The accumulation of the compound G₁ occurred with the decrease of riboflavin formation, exhibiting the maximum amounts at the addition of 0.5% dimeric diacetyl. Therefore, the results demonstrate an inverse correlation between the formation of riboflavin and the accumulation of compound G₁.

4. Identification of the purified compound G₁

Table 1 shows the results of paper chromatographic analysis with isolated

Fig. 3.

Fig. 4.

Fig. 3. Effects of diacetyl monomer, dimer and trimer on riboflavin production. Diacetyl derivatives were supplemented at indicated concentrations to the non-growing cell medium (pH 6.8). After the treatment with vacuum infiltration, the incubation was done in the dark at 27°C for 18 hr. Thereafter, the amounts of riboflavin were respectively determined in the manner described in MATERIALS AND METHODS.

Fig. 4. Effect of dimeric diacetyl on riboflavin formation and the accumulation of green fluorescent compound (compound G₁). Dimeric diacetyl at indicated concentrations was added to the non-growing cell of *E. ashbyii*, accompanied by vacuum infiltration. The incubation was allowed to proceed at 27°C for 18 hr. The riboflavin contents were spectrophotometrically determined and the contents of compound G₁ accumulated were fluorometrically measured by the methods mentioned in MATERIALS AND METHODS.

Table 1. Paper chromatography of compound G₁ and compound G. Paper chromatography was done overnight by the ascending procedure at room temperature with indicated solvents. The fluorescent spots on the paper were located under ultraviolet light.

Solvents ^a	A	B	C	D	E	F	G	H	I
	<i>R_f</i> values								
Compound G ₁	0.03	0.18	0.40	0.71	0.15	0.70	0.44	0.35	0.12
Compound G	0.03	0.19	0.40	0.71	0.15	0.69	0.45	0.35	0.11

^a A, BuOH: EtOH: 2 N NH₄OH (20: 2: 5); B, BuOH: pyridine: H₂O (6: 4: 3); C, BuOH: AcOH: H₂O (4: 1: 5); D, 4% citrate; E, BuOH: EtOH: H₂O (50: 15: 30); F, *t*-BuOH: NH₄OH: H₂O (60: 5: 35); G, *t*-BuOH: H₂O (60: 40); H, BuOH: AcOH: H₂O (3: 2: 2); I, BuOH: 5 N AcOH (7: 3).

compound G_1 and authentic 6,7-dimethyl-8-ribityllumazine (compound G). R_f values of compound G_1 coincide well with those of compound G in the various solvents indicated. Moreover, fluorometric analyses were performed by measurements of excitation and emission spectra of the compound because the method can exclude the interference by the non-fluorescent compounds possibly contained in a trace amount in the compound G_1 preparation. Excitation spectra were obtained by measuring the fluorescence at 530 nm emitted due to the excitation of the compounds with the wavelengths indicated in Fig. 5. The spectra of compound G_1

Fig. 5.

Fig. 5. Excitation spectra of compound G_1 and compound G. For experimental conditions see details in the text.

Fig. 6.

Fig. 6. Emission spectra of compound G_1 and compound G. For experimental conditions see details in the text.

and compound G completely coincided, indicating two absorption maxima at 280 and 408 nm in 0.05 M phosphate buffer (pH 7.0). Furthermore, emission spectra of the compounds were taken by determining the fluorescence at the indicated wavelengths emitted by exciting the compounds at a wavelength of 300 nm, as seen in Fig. 6. The results indicate the good coincidence between the spectra of compound G_1 and compound G. Maximum intensity of fluorescence was observed at 480 nm in 0.05 M phosphate buffer (pH 7.0).

Previously it was reported that the chemical conversion of compound G to 6-methyl-7-hydroxy-8-ribityllumazine (compound V) occurs through the dehydrogenetic demethylation reaction by catalysis of *p*-quinone (12). Thus, a study was made on whether the conversion of the purified compound G_1 to compound V

Fig. 7. Possible formation of 6-methyl-7-hydroxy-8-ribityllumazine (compound V) from compound G_1 due to *p*-quinone. The reaction mixture contained compound G_1 , 0.001 M *p*-quinone and 0.05 M phosphate buffer (pH 7.0) in 3 ml. The reaction was left to proceed at 30°C during the incubation and emission spectra were followed at indicated incubation times by exciting the compounds with a wavelength at 280 nm.

proceeds with the same mechanism as above. Figure 7 shows that the wavelength of the maximum fluorescence of compound G_1 initially observed at 480 nm that gradually shifted to that at 420 nm being characteristic of compound V with increasing reaction times. The results provided evidence that the isolated compound G_1 is 6,7-dimethyl-8-ribityllumazine.

Furthermore, spectrophotometric analysis of compound G_1 was performed with measurements of ultraviolet and visible absorption spectra and of infrared spectra. Figure 8 demonstrates the results of ultraviolet and visible absorption

Fig. 8. Ultraviolet and visible absorption spectra of compound G_1 and compound G. Absorption spectra of compound G_1 and compound G were determined in 0.1 N HCl and 0.1 N NaOH over a wavelength of 210–460 nm.

Fig. 9. Infrared spectra of compound G_1 and compound G on KBr pellets.

spectra in acid and alkaline solutions over a wavelength of 210–460 nm. The spectra of compound G_1 and compound G were quite similar in both solutions. Maximum absorptions were detected at 257, 278 (shoulder) and 408 nm in 0.1 N HCl and 230, 281 and 314 nm in 0.1 N NaOH. The spectra in distilled water and 0.05 M phosphate buffer (pH 7.0) were the same as in the acid solution. Figure 9 represents the infrared spectra of the purified compound G_1 and an authentic compound G measured by a KBr pellet method. Both spectra gave well coincided results

Fig. 10. Possible formation of riboflavin from compound G_1 by the riboflavin synthetase from *E. ashbyii*. The reaction mixture contained a certain amount of compound G_1 , enzyme 0.18 mg, 5 mM Na_2SO_3 , 2.5 mM mercaptoethanol and 2.5 mM EDTA in 50 mM potassium phosphate buffer (pH 6.5) (1 ml). The reaction was initiated with the addition of enzyme and proceeded in the dark at 37°C during the incubation. Absorption spectra at indicated times were respectively measured over a 340–500 nm wavelength.

Table 2. Paper chromatography of the product formed by the riboflavin synthetase reaction. An appropriate volume of the mixture after the reaction done in Fig. 10 was spotted on the paper. Paper chromatography was developed overnight by ascending the method at room temperature with various organic solvents. The spots on the paper were detected under ultraviolet light.

Solvents ^a	A	B	C	D	E	F	G	H	I
	<i>R_f</i> values								
Riboflavin	0.71	0.41	0.35	0.49	0.45	0.42	0.86	0.43	0.31
Product	0.70	0.41	0.35	0.48	0.45	0.42	0.86	0.43	0.32

^a A, BuOH: AcOH: H₂O (3: 2: 2); B, *iso*PrOH: NH₄OH: H₂O (120: 10: 30); C, *t*-BuOH: NH₄OH: H₂O (60: 5: 35); D, *t*-BuOH: H₂O (6: 4); E, BuOH: pyridine: H₂O (6: 4: 3); F, MeOH: HCl: H₂O (60: 5: 35); G, *isopropylether*: 90% HCOOH (9: 6); H, PrOH: NH₄OH: H₂O (6: 3: 1); I, BuOH: EtOH: H₂O (50: 15: 35).

with the peaks in a region of 400 to 1200 cm⁻¹ commonly found in pteridine derivatives.

Furthermore, the result obtained through the riboflavin synthetase reaction is shown in Fig. 10. The spectrum of the reaction mixture continuously changed with increased reaction times and completely transformed to the spectrum having two absorption maxima at 370 and 450 nm over indicated wavelengths at 50 min after the reaction. Moreover, the product in the reaction mixture underwent paper chromatographic analysis, as seen in Table 2. *R_f* values of the product were the same as those of authentic riboflavin in these developing solvents. Thus, the product formed from compound G₁ by riboflavin synthetase is concluded to be riboflavin. Accordingly, compound G₁ appears to be 6,7-dimethyl-8-ribityllumazine based on its enzymic characteristics.

DISCUSSION

In the present paper, the effects of various sugars and carbonyl compounds on riboflavin formation were examined with non-growing cells of *Eremothecium ashbyii*. It was verified that glucose is the most stimulatory sugar but pentoses are not so effective. This possibly suggests that a 4-carbon source, being two building blocks of riboflavin, not a ribityl source is a limiting step in flavinogenesis by *Eremothecium ashbyii*. While, pyruvate, acetate and acetoacetate, metabolites involved in the glycolytic pathway, rigidly inhibited riboflavin formation although glucose as a starting sugar in the pathway markedly stimulated the formation at the concentrations used.

On the other hand, glyoxal, diacetyl and acetoin which were established as trapping agents of possible intermediates in flavinogenesis (1, 13, 14), inhibited riboflavin formation to a great extent except for acetoin.

In the experiment, it is to be noted that ribitol shows no effect on riboflavin formation. Recently, the present authors elucidated that a true nucleotide pre-

cursor is guanosine triphosphate (GTP) (15, 16), which is converted to 6,7-dimethyl-8-ribityllumazine (compound G) after rupture of the imidazole ring with the elimination of C 8 (17). Furthermore, the authors provided evidence that an immediate precursor to compound G is 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (7, 8), having the same structure as that of the byproduct formed in the riboflavin synthetase reaction (18). The intermediate, the byproduct and compound G commonly have ribityl side chains on their molecules. Therefore, in the conversion reaction of GTP to compound G *via* the pyrimidine intermediate, it is quite reasonable to consider that the cleavage reaction of GTP accompanied by elimination of carbon 8 proceeds retaining the ribofuranosyl chain and then the side chain is reduced to form a ribityl side chain although the deamination step of GTP must be taken into account in the course of the reaction. The results obtained in the use of ribitol and pentose derivatives may support the consideration that the ribose moiety of GTP is incorporated without degradation into the ribityl side chain of riboflavin.

On the other hand, a trapping agent, dimeric diacetyl, restricted riboflavin formation at the high concentration and simultaneously caused the accumulation of two green fluorescent compounds (referred to compound G₁ and compound G₂ based on the eluted order from a Dowex 50W × 4 column) inside and outside the cells. The accumulated amounts of compound G₁ were much larger than those of compound G₂. Furthermore, the accumulation of the former increased with increasing additions of dimeric diacetyl in an inverse relation to the formation of riboflavin. The results give some possibilities that the added dimeric diacetyl functioned as a direct inhibitor of any step in the riboflavin biosynthetic pathway or a trapping agent of possible intermediate in flavinogenesis resulting in the deficiency of the intermediate and that the derivative, possibly 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-8-ribityllumazine, from the intermediate and dimeric diacetyl due to its trapping action inhibited the riboflavin biosynthetic pathway.

The problems were partially solved by experiments with the riboflavin synthetase and dimeric diacetyl at varied concentrations. As seen in Table 3, the increased added diacetyl dimer in the reaction mixture gradually inhibited riboflavin formation by the riboflavin synthetase from *E. ashbyii*. Accordingly, it seems possible that dimeric diacetyl itself or the complex of the agent with the byproduct directly inhibits the synthetase reaction in cells. However, the detailed inhibition mechanism of dimeric diacetyl is still unclear. The accumulated compound G₁ was isolated in a large quantity from non-growing cells of *E. ashbyii* and highly purified through many kinds of column chromatography. Moreover, it was established that compound G₁ is 6,7-dimethyl-8-ribityllumazine, by fluorometric analyses with excitation and emission spectra, by emission spectra changed in the presence of *p*-quinone, by spectrophotometric analyses with ultraviolet and visible absorption spectra and infrared spectra and by enzymic analysis with riboflavin synthetase from *E. ashbyii*.

Table 3. Effect of dimeric diacetyl at varied concentrations on the riboflavin synthetase reaction. The reaction mixture (1.5 ml) contained 15 μ moles mercaptoethanol, 15 μ moles Na_2SO_8 , 75 μ moles potassium phosphate (pH 7.0), 225 nmoles 6,7-dimethyl-8-ribityllumazine, dimeric diacetyl at indicated concentrations and the crude enzyme (equivalent to about 1 mg protein). The reaction proceeded at 37°C for 30 min. The termination of the reaction was performed with the addition of 0.5 ml of 30% trichloroacetic acid and 1.0 ml of distilled water, followed by centrifugation. The supernatant was submitted to spectrophotometric determination of riboflavin yields by the method of PLAUT (20). Protein content was determined by the method of LOWRY (21).

Concentration of dimeric diacetyl (mM)	Riboflavin formed (nmol/ml)	Inhibition (%)
0	28.1	0
6	22.9	18.5
16	18.9	32.7
27	14.3	49.1
40	12.0	57.3
53	11.4	59.4

The inhibitor which directly restricts the activity of riboflavin synthetase has not been found so far, except for substrate analogs (19). On this point, the inhibitory mechanism of dimeric diacetyl is of great interest, although it is obscure.

The experiments are being carried out in a later paper to elucidate the structure of another accumulated compound G_2 and its function in the biosynthetic pathway of riboflavin.

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