Summary

1) Sodium lauryl sulfate (SLS) was hydrolysed in acidic solution at a high temperature, and the rate of hydrolysis was dependent on not only H^+ concentration but also SLS concentration. It was recognized that the rate of reaction was increased strikingly in the region of a certain concentration of SLS.

2) When the Critical micelle concentration (CMC) of SLS was determined by solubilization method with dye (Sudan III), it was approximately coincident with region of a certain concentration where the rate of reaction increased strikingly.

3) On the contrary to acidic solution, in alkaline solution, SLS above the CMC was a little more stable than that of below.

(Received December 27, 1961)

UDC 543.544:577.164.12:582.284

5. **Mitsuko Asai and Satoru Kuwada** : Application of Chromatography. XLIV.[*1] Action of an Enzyme from *Eremothecium ashbyii* on 6-Methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)ribolumazine.

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Plaut[1] reported previously that the application of an enzyme from *Ashbya gossypii* to 6,7-dimethylribolumazine (G-compound) resulted in splitting of a four carbon compound from the pyrazine ring, which underwent condensation with 6,7-dimethylribolumazine to form riboflavin. As described in the preceding paper,[*1] the present authors duplicated the experiment using the enzume of *Eremothecium ashbyii* and obtained nearly the same results, but could not give a definite conclusion about the fate of the uracil compound formed by the removal of the four-carbon compound and the formation mechanism of 6-methyl-7-hydroxyribolumazine (V-compound) produced simultaneously in a large quantity.

Birch and Moye[2] previously condensed the diaminouracil (I; R=H) with the aldol compound[3] II, which is a dimer of diacetyl, at acid pH and converted the product III (R=H) into lumichrome by ring closure with alkali. Later, they also applied this reaction to 4-methylamino-5-aminouracil (I; $R=CH_3$) and converted the condensation product III ($R=CH_3$) into lumiflavin by treating with polyphosphoric acid.[4] They also synthesized 6,7,8-trimethyllumazine (IV) and allowed the product to react with diacetyl, but, as the formation of lumiflavin was not observed, they thought that a condensation between the diaminouracil compound and the aldol compound may be more reasonable as the pathway in the biosynthesis of riboflavin than the route which passes through 6,7-dimethylribolumazine.

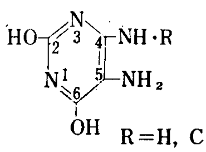
[*1] Part XLIII : This Bulletin, **9**, 503 (1961).

[*2] Juso-nishino-cho, Higashiyodogawa-ku, Osaka (浅井満子, 桑田 智).

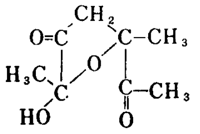
[*3] Cresswell and Wood (loc. cit.) named this compound 7(2-hydroxy-2-methyl-3-oxobutyl)-6-methyl-8-ribityllumazine.

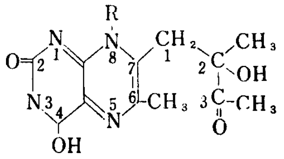
1) G.W.E. Plaut : J. Biol. Chem., **235**, PC 41 (1960).
2) A.J. Birch, C.J. Moye : J. Chem. Soc., **1957**, 412.
3) O. Diels, W.M. Blanchard, H.v.d. Heyden : Ber., **47**, 2359 (1914).
4) A.J. Birch, C.J. Moye : J. Chem. Soc., **1958**, 2622.

More recently, Cresswell and Wood⁵⁾ condensed 4-D-ribitylaminouracil-5-amino (I; R=D-ribityl) with the dimer of diacetyl (II) at pH 4.0 according to the method of Birch, *et al.*, and converted the product, 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)ribolumazine*³ (III; R=ribityl), into riboflavin by heating at pH 1.0. They assumed that there is an increased possibility in the route for riboflavin biosynthesis proposed by Birch and Moye, and concluded that though it was obtained from experiments with a cell-free extract, the views of the present authors⁶⁾ and of Plaut, *et al.*,⁷⁾ that 6,7-dimethylribolumazine may be a precursor in the synthesis of riboflavin in ***Er. ashbyii*** or ***A. gossipii***, do not exclude the existence of a more efficient route.

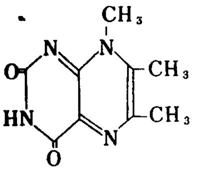


I


II



III


IV

On the other hand, Mitsuda, *et al.* reported the production of riboflavin from 6,7-dimethylribolumazine and a simultaneous formation of 6-methyl-7-hydroxyribolumazine by the action of the enzyme contained in the green leaves of a plant such as the spinach. Recently, Mitsuda, Kawai, and Suzuki⁸⁾ investigated the ratio of these products and attempted to explain the mechanism of biosynthesis of riboflavin on the basis of the view of Birch, *et al.*

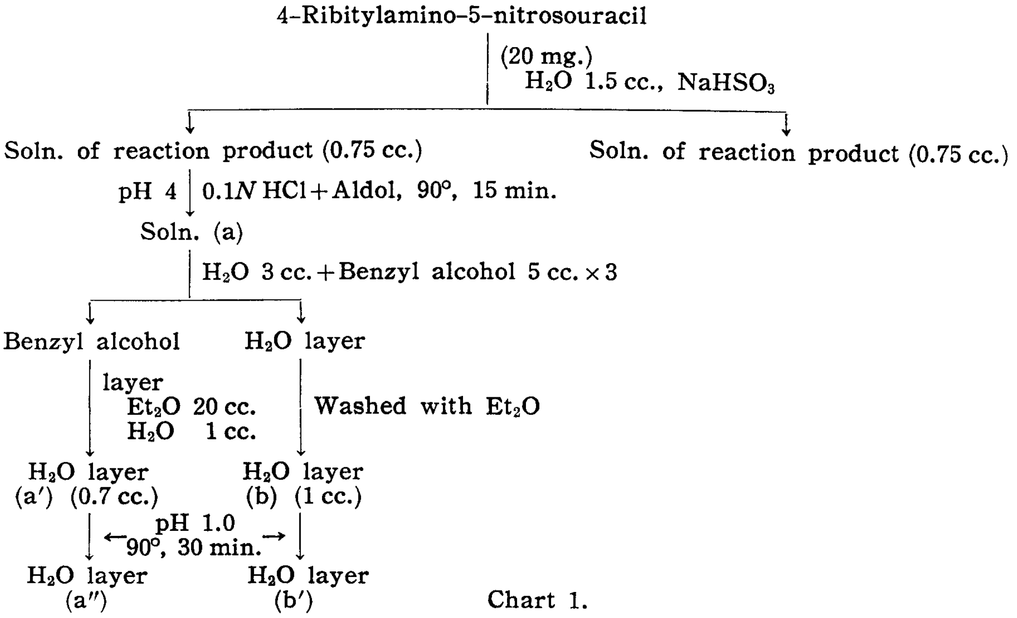


Chart 1.

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5) R. M. Cresswell, H. C. S. Wood: Proc. Chem. Soc., **1959**, 387; J. Chem. Soc., **1960**, 4768.

6) T. Masuda, T. Kishi, M. Asai, S. Kuwada: This Bulletin, **4**, 375 (1956); **5**, 136 (1957); **6**, 619 (1958).

7) G. F. Maley, G. W. E. Plaut: J. Am. Chem. Soc., **81**, 2025 (1959).

8) H. Mitsuda, F. Kawai, J. Suzuki: Vitamins (Japan), **23**, 415 (1961).

For the past several years, the authors have been making some effort to clarify the mechanism in the biosynthesis of riboflavin. Experimental results obtained to date produced a doubt about the route for the biosynthesis of riboflavin as suggested by Birch, *et al.* and by Cresswell, *et al.*, and attempts were made to prepare 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)ribolumazine synthesized by Cresswell and Wood,[5] and apply the enzyme of *Er. ashbyii* on this product to see if riboflavin would be produced as in the case of 6,7-dimethylribolumazine.

The aldol compound II was prepared from diacetyl according to the method of Diels, *et al.*,[3] and the product was reacted at pH 4.0 with the diaminouracil compound obtained by reduction of 4-ribitylamino-5-nitrosouracil[9] with sodium hydrosulfite. A part of the reaction mixture a, per se, was subjected to paper partition chromatography (cf. Chart 1), and the chromatogram was examined under ultraviolet rays to detect the Rf value of the spot, which was in complete agreement with that of 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)ribolumazine observed by Cresswell and Wood.

The reaction mixture a was extracted with benzyl alcohol. The alcohol layer was shaken with ether and water to obtain a greenish yellow aqueous layer a′. The aqueous layer of a was washed with ether and set aside as fraction b. Both fractions a′ and b were chromatographed, and a distinct green spot with Rf 0.16 was detected from fraction a′ and a faint green spot with Rf 0.16 from fraction b, which indicated that almost all the desired product was transferred into the fraction a′. The spot of the desired product was positive to the benzidine reaction and was so unstable in the air that it turned brown immediately.

A part of the fraction a′ was developed on paper, the spot corresponding to Rf 0.16 was extracted with methanol, and an aqueous solution of the residue of the methanol extract, after being adjusted to pH 1.0 with 0.1*N* hydrochloric acid, gave the ultraviolet spectrum shown in Fig. 1. As seen in the figure the spectrum is in good agreement with the spectrum of the lumazine compound III observed by Cresswell and Wood.

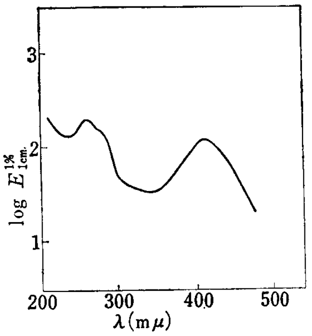

Fig. 1. Ultraviolet Spectrum of 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)-ribolumazine

The fractions a′ and b were adjusted to pH 1.0 with hydrochloric acid and heated, giving the reaciton mixtures a″ and b′, respectively. Paper partition chromatography of these mixtures yielded the results shown in Table I. From this result, the formation of the condensation product III was proved and the pathway of conversion of III to riboflavin was made clear, confirming the report of Cresswell and Wood.

An attempt was further made to apply the crude enzyme solution prepared from *Er. ashbyii* on the remainder of the fraction a′ used for measurement of ultraviolet spectrum to see whether riboflavin is produced or not. However, as will be described in the experimental part, the reaction mixtures obtained by the action of the enzyme solution as such or after inactivating its enzymic activity by heating gave almost the

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[9] T. Masuda, T. Kishi, M. Asai, S. Kuwada: This Bulletin, **7**, 361 (1959).

TABLE I.

Fraction	Solvent system (A) BuOH-5N AcOH (7:3)							Solvent system (B) 3% NH_4Cl				
a	0.04 YG+				0.17 G⧻		0.27 O+	0.31 V+	0.35 Y+	0.39 R+	0.68 G⧺	0.75 Y+
a′	0.05 YG+		0.10 G±	0.12 Y+	0.16 G⧻		0.29 O+					
a″			0.10 G±			0.25 Y⧻						
b	0.04 YG+	0.07 +Y	0.10 Y+		0.16 G±							
b′	0.03 B±		0.10 G±			0.25 Y+						
					↑ Lumazine (III)	↖ Riboflavin						

YG: yellowish green, G: green, R: red, V: violet, Y: yellow,
O: orange, B: blue. ±, +, ⧻: show the intensity of color.

same chromatogram (though it was a little different from that of the original sample), and the spots of riboflavin and 6-methyl-7-hydroxyribolumazine were not detected at all. On the contrary, when 6,7-dimethylribolumazine was reacted with the enzyme solution under the same conditions as above, a clear yellow spot of riboflavin and a purple spot of 6-methyl-7-hydroxyribolumazine were observed as was often reported.

The above results are merely qualitative experimental data, but it may be concluded that, unlike 6,7-dimethylribolumazine, 6-methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)ribolumazine is not converted to riboflavin and 6-methyl-7-hydroxyribolumazine *in vitro* by the action of an enzyme from the mycelium of *Er. ashbyii.*

Experimental

I Synthesis of 6-Methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)ribolumazine (III)——To a mixture of 100 cc. of diacetyl and 400 cc. of distilled H_2O 350 cc. of N KOH was added dropwise over a period of about 1 hr., under cooling with ice H_2O. The mixture was made slightly acid with H_2SO_4 and extracted with Et_2O in a liquid extractor for about 10 hr. The residue from Et_2O extract was fractionated to give a fraction of b.p$_{0.3}$ 110~120°. Diels *et al.*[3] regarded this fraction as the dimer of diacetyl and named it the aldol compound II.

A solution of 20 mg. of 4-ribitylamino-5-nitrosouracil[9] in 1.5 cc. of distilled H_2O was reduced with ca. 10 mg. of $NaHSO_3$ until it was decolorized, 0.75 cc. of the resulting solution was adjusted to pH 4.0 with 0.1N HCl, and heated with 0.1 cc. of the above aldol compound at 90° for 15 min., giving the reaction mixture a. Results obtained in a similar reaction but at another pH will be reported elsewhere.

A part of the reaction mixture a was developed on paper with A (BuOH-5N AcOH, 7:3) and B (3% aqueous solution of NH_4Cl), and the chromatograms were examined under a UV, the results being shown in Table I. According to Cresswell and Wood,[5] the Rf value of the title compound is 0.17 when developed with the solvent A and 0.65 when developed with the solvent B, but mention was not made on its color. The reaction mixture was diluted with 3 cc. of distilled H_2O and extracted with three 5 cc. portions of benzyl alcohol. The alcohol layer was shaken with 20 cc. of Et_2O and 1 cc. of H_2O, and the separated aqueous layer (0.7 cc) was set aside as fraction a′.

The former aqueous layer b and a′ were developed on paper with the solvent A to give the chromatograms shown in Table I and it was found that lamost all the desired lumazine compound III was transferred into fraction a′. The spot at Rf 0.16 was positive to the benzidine reaction and easily turned brown in the air.

About 0.3 cc. of fraction a′ was applied on one end of a thick filter paper strip, 4×40 cm. (Toyo Roshi No. 526) and developed with the solvent A. The part of the strip corresponding to Rf 0.16~0.17 was cut out, extracted with three 5-cc. portions of abs. MeOH at room temperature, and extract was evaporated, leaving 1.3 mg. of a residue. The residue was dissolved in 0.5 cc. of distilled H_2O, 0.1 cc. of the solution was adjust to pH 1.0 with 9.9 cc. of 0.1N HCl, and its UV spectrum was measured, giving the absorption curve shown in Fig. 1. The curve has three maxima at 258, 276 (shoulder), and 411 mμ, and this is in complete agreement with the data of Cresswell and Wood.[5]

The above-mentioned fractions were prepared by the method shown in Chart 1.

II Formation of Riboflavin from the Above-mentioned Condensation Product——An amount of 0.3 cc. each of the aqueous solutions a′ and b was adjusted to pH 1.0 with HCl and heated at 90° for 30 min. The solution a″ and b′ (see Chart 1) obtained respectively from a′ and b, were subjected to paper partition chromatography to give the chromatograms shown in Table I. The yellow fluorescent spot at Rf 0.25 was extracted with 0.1*N* HCl, and as the UV spectrum of the extract exhibited maxima at 223, 267, 376, and 445 mμ, the spot was confirmed to be that of riboflavin.

III Action of Enzyme of *Eremotheciun ashbyii* upon 6-Methyl-7-(2-hydroxy-3-methyl-3-oxobutyl)-ribolumazine——The reaction mixture a′ obtained as described in I was separated by paper partition chromatography, and the spot corresponding to the desired compound was extracted with MeOH. The extract was evaporated, a part of the residue was used for measurement of UV spectrum, and 0.1 cc. of the remainder was diluted with 0.9 cc. of distilled H_2O. The solution was incubated with 0.1 cc. of a crude enzyme solution, obtained from *Er. ashbyii*, at 37° for 1 hr., giving the reaction mixture 1. Control solution was the same as above, but the enzyme solution added was heated beforehand, to give the reaction mixture 2. In paralled with this, 0.1 cc. of the enzyme solution was added to 0.1 cc. of a 0.02% aqueous solution of 6,7-dimethylribolumazine, and the mixture was incubated under the same conditions as above to give the reaction mixture (3). The three reaction mixtures were submitted to paper partition chromatography, yielding the chromatograms shown in Table II.

Table II.

Sample	Rf value Solvent system : EtOH-BuOH-HO (15:50:35)							Rf value Solvent system (A) : BuOH-5*N* AcOH (7:3)					
1	0.17 G ±		0.23 Y ++				0.31 O +	0.05 Y G +		0.12 Y ++			0.26 O +
2	0.17 G ±		0.23 Y ++		0.27 B +		0.31 O +	0.05 Y G +			0.13 Y ++		0.26 O +
3		0.20 G +		0.25 V ++		0.30 Y +++			0.08 G B +			0.25 Y +++	
				↑ 6-Methyl-7-hydroxy-ribolumazine		↖ Riboflavin						↑ Riboflavin	

As seen from this Table, both reaction mixtures 1 and 2 showed nearly the same chromatgram and did not exhibit the spot of 6-methyl-7-hydroxyribolumazine or riboflavin, though they were prepared by incubation with the enzyme solution as it was or after previous heating. In both chromatograms, the green fluorescent spot of the lumazine compound III had disappeared and a new yellow spot Rf 0.12, solvent system A; Rf 0.23, solvent system C was detected. The lumazine compound III was left standing at 10° overnight without addition of the enzyme solution, but as the chromatogram showed the spot of the compound clearly, this compound was found not to be converted by the enzymic reaction *in vitro* to 6-methyl-7-hydroxyribolumazine and riboflavin in an amount detectable by paper partition chromatography.

On the contray, when 6,7-dimethylribolumazine was incubated with the enzyme solution under the above-mentioned conditions, the spots of riboflavin and 6-methyl-7-hydroxyribolumazine were clearly detected as has hitherto been reported frequently.

Summary

6-Methyl-7-(2-hydroxy-2-methyl-3-oxobutyl)ribolumazine (III) was synthesized by the method of Cresswell and Wood,[5] and the product was found to be converted to riboflavin at pH 1.0, but action of the enzyme of *Eremothecium ashbyii* on III *in vitro* produced neigher riboflavin nor 6-methyl-7-hydroxyribolumazine.

(Received January 5, 1962)