U.D.C. 577.164.12 : 582.284

12-5

20. **Toru Masuda** : Application of Chromatography. XXXII.* Biosynthesis of Riboflavin by *Eremothecium ashbyii*.

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In Parts XXIX⁽¹⁾ and XXXI⁽²⁾ of this series the author presumed the structure of the green fluorescent substance, G compound, isolated from the mycelium of *Er. ashbyii* to be 8-ribityl-6,7-dimethyllumazine from its absorption spectrum and photodecomposition products.

If this structure assigned to G compound is correct, it is a compound which resembles riboflavin but lacks A-ring and will produce riboflavin by dehydrative condensation with one mole of diacetyl. Therefore, attempts were made to condense G compound with diacetyl or with acetoin, a reduction product of diacetyl. In the case of diacetyl, the condensation was effected merely on heating the materials in a test tube, while the condensation with acetoin took place only in the presence of a little piperidine. The results support the proposed structure of G compound. It would be too early to conclude that the reaction in the test tube was effected by the same mechanism as in the mycelium of *Er. ashbyii*, but examination of the mycelium and the culture filtrate showed a large quantity of acetoin, which can condense with G compound to form riboflavin.

Quantitative determinations of riboflavin, G compound, and acetoin in the mycelium and the culture filtrate indicated that they exist in a ratio of about 100:2∼5:10, and the proportion of G compound increases with the age of the culture.

In the present report consideration was also made of the mechanism of biosynthesis of riboflavin through G compound.

Experimental

Reaction between G Compound and Diacetyl—Ten mg. of G compound was heated with 2 cc. of diacetyl at 120∼130° in an oil bath, by which the former dissolved gradually to form a brown solution after 6 hrs. The reaction mixture was subjected to paper partition chromatography, using various solvents. The results are shown in Table I. An attempt to react G compund with acetoin instead of diacetyl under the same conditions was unsuccessful, but they seemed to react to some extent on addition of 0.1 cc. of piperidine. The results of paper chromatography of the reaction mixture are also shown in Table I.

Separation of Riboflavin from the Reaction Mixture containing G compound and Diacetyl, and Its Identification—0.05 cc. of the above reaction mixture was applied to a strip of Toyo Roshi No. 5B, 8×44 cm., and developed with $AcOH \cdot BuOH \cdot H_2O$ (1:4:5). The yellow fluorescent band detected at the site corresponding to the Rf of riboflavin was cut out and extracted with 10 cc. of water. As shown in Fig. 1, the ultraviolet spectrum of the extract was in complete agreement with that of riboflavin. The amount of riboflavin, calculated from the absorption coefficient at 445 mμ, was about 6 γ in 1 cc. of the extract. It follows from this that 2.4 mg. of riboflavin was formed in the reaction.

On the other hand, 1 cc. of the extract was diluted with water to 50 cc. and subjected to bioassay, using *L. casei* as the test microörganism. The standard curve (37°, 40 hrs.) used in this assay is given in Fig. 2. The amount of riboflavin in 1 cc. of the sample, calculated by this method was, 0.10 γ, which is in close agreement with the figure, 0.12 γ, calculated from the absorption coefficient at 445 mμ. G compound itself was inactive to the assay.

Identification of Acetoin in the Mycelium of *Er. ashbyii*—A mixture of 10 g. of the mycelium

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* This constitutes a part of a series entitled "Application of Chromatography" by Satoru Kuwada. Part XXXI. This Bulletin, **5**, 28(1957).

** Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (増田 亨).

1) T. Masuda : This Bulletin, **4**, 375(1956).

2) T. Masuda : *Ibid.*, **5**, 28(1957).

TABLE I.

Sample / Developing Solvent	Rf (Fluorescence)							
	G compound + Diacetyl					G compound	Riboflavin	6,7-Dimethyllumazine
BuOH•EtOH•H_2O	0.23	0.35	0.40	0.53	0.61	0.23	0.40	0.53
(50:15:35)	G++	YG+	Y++	GB+	±	G	Y	GB
AcOH•BuOH•H_2O	0.13	0.22	0.28	0.44	0.54	0.13	0.27	0.44
(1:4:5)	G++	YG+	Y++	GB+	Y ±	G	Y	GB
Pyridine•BuOH•H_2O	0.53	0.65	0.71			0.53	0.65	0.71
(3:4:7)	G++	Y +	GB+			G	Y	GB
	G compound + Acetoin + Piperidine							
BuOH•EtOH•H_2O	0.23	0.35	0.40	0.52				
(50:15:35)	G++	YG++	Y +	GB+				
AcOH•BuOH•H_2O	0.13	0.22	0.28	0.44				
(1:4:5)	G++	YG++	Y +	GB+				
Pyridine•BuOH•H_2O	0.53	0.65	0.71					
(3:4:7)	G +	Y +	GB+					

Color of Fluorescence : G = green, YG = yellowish green, GB = greenish blue, Y = yellow.
Intensity of Fluorescence : ±, +, ++.

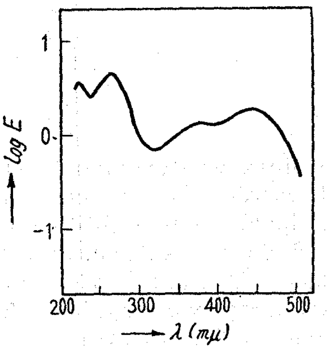

Fig. 1.

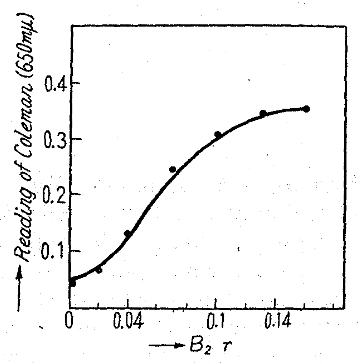

Fig. 2.

obtained from a 60-hr. culture of *Er. ashbyii* and 100 cc. of water was heated in a distilling flask in an oil bath, and about 30 cc. of the forerun was collected in a cooled receiver. 2 cc. of the distillate was subjected to the test for diacetyl by the method of White, *et al.*,[3] using hydroxylamine and urea, but the result was negative. Then, 10 g. of the mycelium was heated with 5 cc. of 50% $FeCl_3$ solution and 1 cc. of conc. H_2SO_4 as above, and the distillate was treated in the same way to give a positive result. From this fact it is obvious that diacetyl was present in the mycelium in the form of acetoin.

Next, 85 g. of the mycelium was treated as above with 15 cc. of 50% $FeCl_3$ solution and 3 cc. of conc. H_2SO_4, about 30 cc. of the distillate was heated with diaminouracil, and the concentrated reaction mixture was developed on filter paper with BuOH•EtOH•H_2O(50:15:35), whereupon a bluish green fluorescent band with Rf 0.55 was detected. This Rf is in complete agreement with that of 6,7-dimethyllumazine synthesized from diacetyl and diaminouracil. It is certain from this that the distillate contained diacetyl.

Determination of Acetoin—A mixture of 15 g. of the mycelium, 100 cc. of water, 1 cc. of conc. H_2SO_4, and 5 cc. of 50% $FeCl_3$ solution was heated in a distilling flask in an oil bath. Boiling stones and some octanol were also added to make the distillation smooth. About 40 cc. of the distillate was collected in an ice-cooled 50-cc. measuring cylinder and then diluted with water to 50 cc. 2 cc. of the diluted distillate was heated in a test tube with 1 cc. of 1% hydroxylamine solution, 1 cc. of 3% urea solution, and 2 cc. of a mixture of conc. H_2SO_4 and 89% H_3PO_4(1:3 by volume) in a water bath for 30 mins. After cooling, the mixture was diluted with water to 10 cc. and its absorption coefficient at 480 mμ was measured with the Beckman spectrophotometer. As a standard solution, a solution of diacetyl containing 100 γ/cc. was prepared immediately before use and treated as above. The amount of acetoin was calculated from the value of the standard measurement. The results are shown in Table II.

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[3] A.G.C. White, L.O. Krampitz, C.H. Werkman : Arch. Biochem., **9**, 229(1946).

TABLE II. Amount of Acetoin, G compound, and Total Riboflavin in the Mycelium

Sample No.	Duration of culture hr.	Wt. of wet mycelium g./L.	Acetoin		G compd.		Total riboflavin	
			γ/g.	γ/L.*	γ/g.	mg./L.*	γ/g.	mg./L.*
1	24	6.3	17.5	111				
2	36	20.4	30	615	16.3	0.33	816	18.3
3	48	23.6	60	1410	173.5	4.1	5750	136
4	60	26.2	97.5	2550	298.5	7.8	9000	236
5	72	32.4	136	4450	343	11.1	9040	292
6	84	30.0	136	4100	367.5	11.1	10320	310
7	90	27.2	164	4450	391	10.6	9760	265

* The values were obtained by converting the values in 1 g. of the mycelium into those in the mycelium contained in 1 L. of the culture broth.

When the culture filtrate was used as the sample, 100 cc. of the material was treated in the same manner as above. The result is shown in Table III.

TABLE III. Amount of Acetoin and Total Riboflavin in the Culture Filtrate

Sample No.	Duration of Culture (hr.)	Acetoin (mg./L.)	Total riboflavin (mg./L.)
1	24		
2	36		
3	48	8.55	151
4	60	14.65	190
5	72	27.2	212
6	84	41.6	264
7	90	40.0	310

Determination of G Compound—Five grams of the mycelium was extracted with water at 80° until the extract no longer colored yellow. The combined extracts were concentrated to 10 cc. under reduced pressure, during which time the precipitated riboflavin was filtered off. 0.05 cc. of the concentrated extract was applied to each of four strips of Toyo Roshi No. 5B (4×45 cm.), 7 cm. from the end, and developed with $BuOH \cdot EtOH \cdot H_2O$ (50:15:35). The strips were dried in a dark room and the green fluorescent bands detected under ultraviolet rays were cut out. The bands were extracted with 5 cc. of water in a test tube and the absorption coefficicent at 410 mμ of the extract was measured. Since the absorption coefficient at 410 mμ of G compound is $E^{0.01\%}_{1\,\text{cm.}}$ 3.15, the amount of G compound in the sample can be easily calculated from the value obtained.

G Compound could scarcely be detected in the culture filtrate collected in the early stage of the culture and the content of G compound in the culture filtrate obtained after autolysis of the mycelium was too small to be determined, though it could be detected by paper chromatography.

Determination of Total Riboflavin—Determination of total riboflavin in the mycelium and the culture filtrate was carried out according to the method described in Part XXVII.[4] The results are shown in Tables II and III.

Discussion

When diacetyl or acetoin was reacted with the green fluorescent substance, G compound, which was isolated from the mycelium of *Er. ashbyii* and presumed to be 8-ribityl-6,7-dimethyllumazine, and the reaction mixture was developed on filter paper, a yellow fluorescent band was detected on the chromatogram at the site corresponding to the Rf of riboflavin. From the absorption coefficient and bioassay of its extract, the band was ascertained to be that of riboflavin. From this fact it was found that the structure assigned to G compound is correct and that the two methyl groups at 6 and 7 positions of G compound react easily with the ketone group of diacetyl. On this basis the mycelium and the culture filtrate were investigated and an appreciable amount of acetoin was detected in them.

There are many reports on the biosynthesis of riboflavin, but no paper has been published on the isolation of the intermediate.

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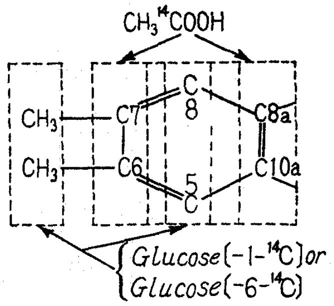
4) T. Masuda, Y. Sawa, M. Asai: This Bulletin, **3**, 375(1955).

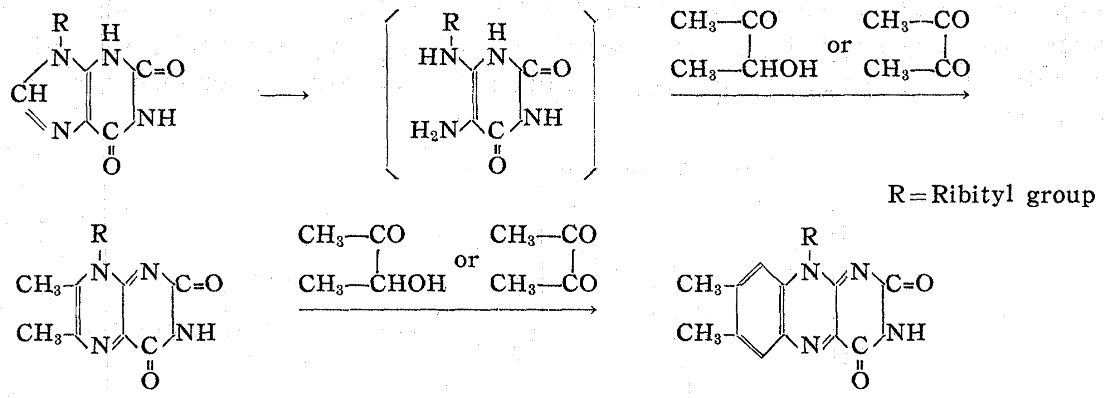
McLaren,[5] McNutt,[6,7] Goodwin,[8,9] and Plaut[10,11] have announced that addition of purine, pyrimidine, or amino acid to the culture medium increased the yield of riboflavin. They also added various radioactive compounds to the medium and investigated the degradation products of the resulting riboflavin to find in what position of the riboflavin the active carbon was incorporated. From these reports it was roughly ascertained that the pyrimidine ring of xanthine and adenine is utilized in the C-ring of riboflavin. The experiment with *Ashbya gossypii* conducted by Plaut[11] revealed that when CH_3-^{14}COOH was added to the medium, ^{14}C was incorporated in positions 6, 7, 8a, and 10a of A-ring, while ^{14}C of glucose[1-^{14}C] or glucose[6-^{14}C] was utilized in positions 5 and 8 and in the methyl groups.



Goodwin[8] once presumed that threonine and serine might be utilized to build the A-ring of riboflavin, but later studies[12] with radioactive serine indicated that serine cannot enter the A-ring.

As mentioned before, the author found that *Er. ashbyii* produces 8-ribityl-6,7-dimethyllumazine and acetoin and that both compounds react to form riboflavin merely by heating. It is not clear yet whether the same reaction occurs in the mycelium, but it is reasonable to believe that such a reaction as this may be accelerated by various enzymes in the living body.

Considering thus, the mechanism of the biosynthesis of riboflavin by *Er. ashbyii* may be presumed to be as follows :



G Compound

Riboflavin

According to this scheme, it is conceivable that ^{14}C of the radioactive glucose is incorporated at the 5 and 8 positions and the methyl groups, and ^{14}C of CH_3-^{14}COOH enters the 6, 7, 8a, and 10a positions. Further, the assumption of Plaut that acetoin may be produced through glucose and pyruvate is also supported by this presumption.

Determination of riboflavin, acetoin, and G compound in the mycelium and the culture filtrate is described in the experimental part and the content of the three compounds in 1 g. of the mycelium is shown in Fig. 3. Riboflavin generally appears from 36 hrs. after start of the culture and gradually increases to reach a maximum at

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5) J. A. McLaren : J. Bacteriol., **63**, 233(1952).
6) W. S. McNutt : J. Biol. Chem., **210**, 511(1954).
7) W. S. McNutt : *Ibid.*, **219**, 365(1956).
8) T. W. Goodwin, S. Peudlington : Biochem. J. (London), **57**, 631(1954).
9) E. G. Brown, T. W. Goodwin, S. Peudlington : *Ibid.*, **61**, 37(1955).
10) G. W. E. Plaut : J. Biol. Chem., **208**, 513(1954).
11) G. W. E. Plaut : *Ibid.*, **211**, 111(1954).
12) T. W. Goodwin, O. T. G. Jones : Biochem. J.(London), **64**, 9(1956).

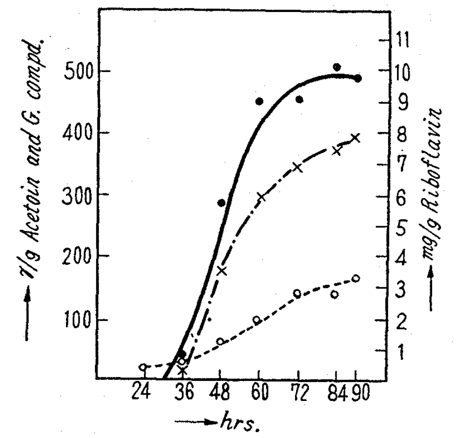

Fig. 3.

Composition of the Mycelium

the 84th hour and then decreases a little, during which time G compound also increases taking a similar course, but its amount is about 1/35 of that of riboflavin at the 48th hour and 1/27 at the 84th hour. That is, the proportion of G compound to riboflavin increases gradually as the culture ages. Since, however, part of the riboflavin in the mycelium dissolves out into the liquid portion, the ratio between the two substances in the whole culture broth may be somewhat different from that in the mycelium. Fig. 4 shows the content of these compounds in 1 L. of the culture broth. As seen from Fig. 4, the content of G compound in the culture broth is 1/70 of that of riboflavin at the 48th hour and 1/50 at the 84th hour. According to results in several experiments, the content of riboflavin reaches a maximum in 70~90 hrs. after start of the culture, and G compound stops increasing at the 72nd hour. On the other hand, the content of acetoin is very small in the mycelium and considerably larger in the culture filtrate, reaching 4~5 times as much as G compound. Several experiments gave about the same result.

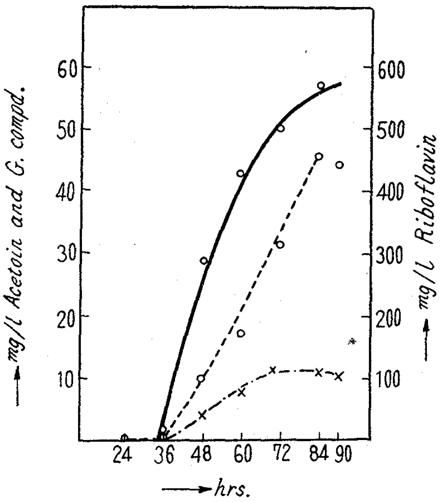

Fig. 4.

Component of the Culture Broth

It would be premature to conclude from the above results that G compound is an intermediate in the biosynthesis of riboflavin, but the fact that, compared with riboflavin, G compound is always detected in small quantities can be understood by assuming that the acetoin produced in large quantities immediately reacts with G compound to produce riboflavin.

The author thanks Mr. Minoru Goto and Mr. Yutaka Shiraishi for their performance of the bioassays and cultures, respectively, and also Mr. Toyokazu Kishi and Mrs. Mitsuko Asai for their help throughout the present work.

Summary

It was ascertained by paper partition chromatography, ultraviolet spectrum, and bioassay with *L. casei* that the green fluorescent substance, G compound, isolated from the mycelium of *Er. ashbyii* readily reacts with diacetyl or with acetoin to form riboflavin. These results established the structure 8–ribityl–6,7–dimethyllumazine, formerly proposed by the author for the G compound.

On the other hand, a large quantity of acetoin was detected in the mycelium and the culture filtrate, and comparing the amount with those of riboflavin and G compound in these materials, the assumption was made that the biosynthesis of riboflavin by *Er. ashbyii* might be effected by condensation of acetoin with G compound at the position of the methyl groups.

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21. **Naofumi Ōi, Kazuko Kageyama, and Keiichiro Miyazaki**: Studies on Intermolecular Bonds by Infrared Absorption Spectra. I. Hydrogen Bonding Power of Antipyrine and Aminopyrine.

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It is well known that antipyrine and aminopyrine form molecular addition compounds with various phenol derivatives or barbital derivatives, but the structure of their molecular compounds are not always obvious.

Hirayama, *et al.*[1] studied solvent effect on the ultraviolet absorption spectra of antipyrine and aminopyrine, and presumed that they form hydrogen bond with solvents, such as $C=O\cdots H$, because their spectra show a remarkable blue shift.

However, detailed studies on how the hydrogen bond is formed between antipyrine or aminopyrine and phenol derivatives have not been made yet.

Useful data concerning this question may be obtained from infrared absorption measurements of certain ternary solutions, each of which is composed of a large quantity of nonpolar solvent (such as carbon tetrachloride) and small quantities of two polar solutes (a proton–donor containing X–H, such as phenol, and a proton–acceptor containing Y, such as antipyrine) and in which complex molecules with the hydrogen bond $X-H\cdots Y$ are present.

Therefore infrared absorption spectra of various ternary solutions were measured in the region of $3000\sim4000\,cm^{-1}$ and $1500\sim2000\,cm^{-1}$. The compositions of ternary solutions examined were phenol, thymol, and 1–naphthol each with antipyrine and carbon tetrachloride, and phenol, thymol, and 1–naphthol, each with aminopyrine and carbon tetrachloride.

For comparison, infrared absorption measurements were made also of binary solutions with compositions of phenol, thymol, 1–naphthol, antipyrine, and aminopyrine, each with carbon tetrachloride.

In these ternary solutions, very similar spectra were obtained and two examples of such spectra are shown in Figs. 1 and 2.

These figures show how the absorption of antipyrine or aminopyrine is affected by

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[1] H. Hirayama, T. Kubota: Ann. Rept. Shionogi Research Lab., **1**, 53(1951).