Summary

Since the reaction of sodium methoxide with 4-chloro-6-methylpyrimidine-2-carbonitrile afforded 2,4-dimethoxy-6-methylpyrimidine, the cyano group in 2- and 4-positions of the pyrimidine ring is fairly active to nucleophilic reagents. Reaction with 2-cyanopyrimidine derivatives showed that the substitution of cyano group with alkoxide ion decreased in the order to primary, secondary, and tertiary alkyls. This reaction did not take place when the alkoxide ion was replaced with phenoxide or thiophenoxide ion, forming acid amide alone. Application of sodium ethoxide to 2-cyanoquinoline, 4-cyanoquinoline, and 4-cyanoacridine failed to produce any ethoxylated derivatives.

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95. Toyokazu Kishi, Mitsuko Asai, Toru Masuda, and Satoru Kuwada : Application of Chromatography. XXXIX.*1 Biosynthesis of Riboflavin. (3).

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One of the present authors (Masuda)[1] once presumed the mechanism of the biosynthesis of riboflavin by *Eremothecium ashbyii* to be as shown in Chart 1. Katagiri, *et al.*[2] and the present authors[3] later made it clear that riboflavin (IV) can be readily prepared from 6,7-dimethylribolumazine (III) by the action of the enzyme of *Er. ashbyii*. As reported in the previous paper*1 the authors synthesized 4-ribitylamino-5-aminouracil (II) as an intermediate of (III) or 6-methyl-7-hydroxyribolumazine and isolated it as crystalline hydrochloride and hydrosulfite. In the present work, an attempt was made on the synthesis of riboflavin (IV) from (II) via (III) by the action of the above-mentioned enzyme.

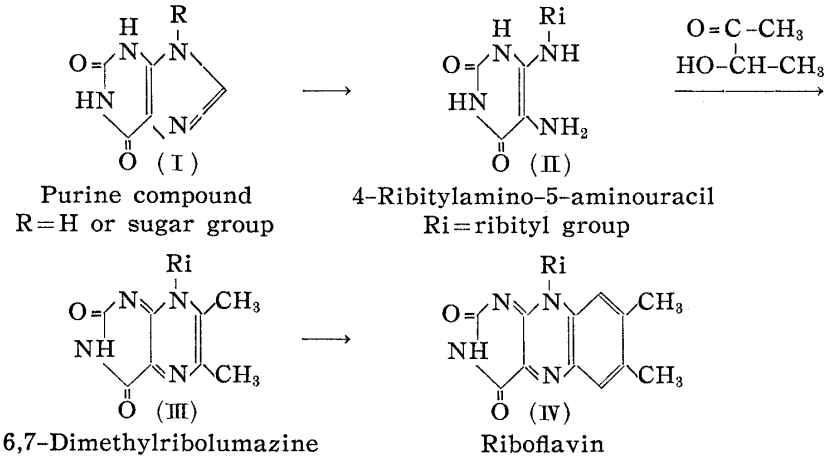

Chart 1.

The authors are grateful to Mr. Minoru Uchida and Dr. Masao Isono for their cooperation in the shaking culture of *Er. ashbyii*.

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*1 Part XXXVIII : This Bulletin, **7**, 366(1959).
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1) T. Masuda : This Bulletin, **5**, 136(1957).
2) H. Katagiri, I. Takeda, K. Imai : J. Vitaminology (Japan), **4**, 285(1958).
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Experimental

(1) **Preparation of Samples**

(a) **Preparation of a Crude Enzyme Solution from *Er. ashbyii*—**The solution was prepared by the method reported in a previous paper[3] using the mycelium obtained by the shaking culture of *Er. ashbyii* for 64 hr.

(b) **Hydrochloride and Hydrosulfite of 4-Ribitylamino-5-aminouracil—**They were prepared according to the method reported previously.*1 Hydrochloride, $C_9H_{16}O_6N_4 \cdot HCl \cdot H_2O$, m.p. 90° (decomp.). Hydrosulfite, $C_9H_{16}O_6N_4 \cdot \frac{1}{2} H_2SO_3$, m.p. 181° (decomp.).

(c) **Acetoin—**This compound was prepared by reducing diacetyl with Zn and 10% H_2SO_4 by the usual method and the fraction of b.p. 140~147° was collected.

(2) **Experimental Method—**As reported in a previous paper,[3] a mixture of the enzyme solution and the substrate was incubated at 37°, and after the lapse of a period of time the products were separated by paper chromatography without being subjected to heat treatment beforehand. The content of 6,7-dimethylribolumazine was too low to be determined by the absorption at 410 mμ and, therefore, only riboflavin was determined. The control was prepared by using the same, but heat-treated enzyme solution. Formation of 6,7-dimethylribolumazine from (II) by the action of the enzyme was confirmed by the following experiment.

A mixture of 19 cc. of the crude enzyme solution, 0.1 cc. of acetoin, and 15 mg. of 4-ribitylamino-5-aminouracil hydrosulfite was incubated at 37° for 3 hr. and the reaction mixture was shaken with benzyl alcohol. The benzyl alcohol layer was shaken with ether and a little water, and the separated aqueous layer was submitted to paper partition chromatography, with the solvent system of EtOH·BuOH·H_2O (15:50:35), pyridine·BuOH·H_2O (4:6:3), or AcOH·BuOH·H_2O (1:4:5). The sample for measurement of UV spectrum was prepared by cutting out from the chromatogram the green fluorescent spot corresponding to the Rf value of 6,7-dimethylribolumazine and extracting it with hot water. Mixtures of 19 cc. of water, 0.1 cc. of acetoin, and 15 mg. of 4-ribitylamino-5-aminouracil hydrosulfite, and of 19 cc. of the crude enzyme solution alone were also treated in the same manner as above.

(3) **Results—**Addition of only 4-ribitylamino-5-aminouracil salt to the enzyme solution produced only a very small amount of riboflavin despite the change of incubation time, quantity of the enzyme solution, and kind of the developing solvent, and therefore it is difficult to assume that riboflavin was biosynthesized in these methods.

Table I. Comparison of Quantities of Riboflavin formed in Two Cases

Amt. of riboflavin detected (γ)		Amt. of riboflavin formed (γ)
	Mean value	
1.54 (R = 0.10) *1		0.10
2.54 (R = 0.20) *2		1.07 ($2.8 \times 10^{-9} M$ (1.4%))
Control		
1.51 (R = 0.10) *3	1.47 (R = 0.15)	
1.44 (R = 0.05) *4		

*1 Mean value of 3 experiments in which no acetoin was added.

*2 Mean value of 3 experiments in which 0.1 cc. of a $10^{-2} M$ acetoin solution was added.

*3 Mean value of 2 experiments in which 4-ribitylamino-5-aminouracil hydrosulfite and acetoin were added.

*4 Mean value of 2 experiments.

R : Maximum value minus minimum value.

In one, 0.1 cc. of $10^{-2} M$ acetoin solution was added to a mixture of 0.1 cc. of the crude enzyme solution (dialyzed for 72 hr.) and 0.1 cc. of $2.05 \times 10^{-3} M$ 4-ribitylamino-5-aminouracil hydrosulfite solution. No acetoin was added in the other.

The content of protein in the enzyme solution was 190 γ/cc. of protein N and the incubation was continued for 3 hr. at 37°. The yield of riboflavin increased by 1.4% when 4-ribitylamino-5-aminouracil hydrosulfite and acetoin were added, and a green fluorescent spot was found at the place corresponding to the Rf value of 6,7-dimethylribolumazine on the chromatogram, but no increase of the yield was observed in the absence of acetoin.

From the result of Table II, pyruvic acid does not seem to take part in the reaction as a carbon donor.

Judging from the result of Table III, acetic acid, acetaldehyde, and formic acid, like pyruvic acid, do not seem to take part in the reaction as carbon donor.

All the reaction mixtures showed yellowish green fluorescence, but the fluorescence of the reaction mixture without enzyme solution was not extracted into benzyl alcohol. Further, paper chromatogram of the latter mixture gave no fluorescent spot at the places corresponding to the Rf values of 6,7-

Table II.

Subst. added (carbon donor)	Amt. of riboflavin detected (γ)	Amt. of riboflavin formed (γ)
Pyruvic acid	1.61 (R = 0.05) *1	0.06
Acetoin	2.82 (R = 0.05) *1	1.22 (3.2×10^{-9} (1.2%))
Control		
Pyruvic acid	1.55 (R = 0.01) *2	
Acetoin	1.60 (R = 0.02) *2	

Incubation was continued for 3 hr. at 37°.

*1 Mean value of 3 experiments.

*2 Mean value of 2 experiments.

0.1 cc. of $10^{-2}M$ acetoin or pyruvic acid solution was added to a mixture of 0.1 cc. of the crude enzyme solution (dialyzed for 72 hr.) and 0.1 cc. of $2.68 \times 10^{-3}M$ 4-ribitylamino-5-aminouracil hydrosulfite solution.

Table. III.

Subst. added (carbon donor)	Amt. of riboflavin detected (γ)	Amt. of riboflavin formed (γ)
Acetoin	3.40 (R = 0.04) *	0.95 ($2.5 \times 10^{-9}M$ (1.1%))
Acetic acid	2.54 (R = 0.07) *	0.1
Acetaldehyde	2.40 (R = 0.04) *	
Formic acid	2.54 (R = 0.07) *	0.1
Control	2.45 (R = 0.14) *	

Incubation was continued for 3 hr. at 37°.

* Mean value of 2 experiments.

0.1 cc. of a $10^{-2}M$ carbon-donor solution was added to a mixture of 0.1 cc. of the crude enzyme solution (dialyzed for 48 hr.) and 0.1 cc. of $2.3 \times 10^{-3}M$ 4-ribitylamino-5-aminouracil hydrosulfite solution.

Table IV. Enzymatic Synthesis of 6,7-Dimethylribolumazine (Rf value)

Reaction system \ Developing solvent	EtOH·BuOH·H_2O (15:50:35)	Pyridine·BuOH·H_2O (4:6:3)	AcOH·BuOH·H_2O (1:4:5)
Enzyme solution + acetoin + 4-ribitylamino-5-aminouracil	0.20 G +	0.22 G +	0.13 G +
	0.40 Y ##	0.42 Y ##	0.32 Y ##
Enzyme solution alone	—	—	—
	0.40 Y +	0.42 Y +	0.32 Y +
H_2O + acetoin + 4-ribitylamino-5-aminouracil	—	—	—
	—	—	—

Incubation was continued for 3 hr. at 37°.

Color designation : G, green fluorescence, Y, yellow fluorescence, + intensity

dimethylribolumazine and riboflavin. Although the enzyme solution used in the reactions exhibited no green spot on the paper chromatogram, it gave a green spot when incubated with 4-ribitylamino-5-aminouracil and acetoin, and the originally observed yellow fluorescent spot markedly increased in intensity in this case. The UV-spectrum of the former spot was in good agreement with that of 6,7-dimethylribolumazine as shown in Fig. 1, and the latter spot was confirmed to be due to riboflavin

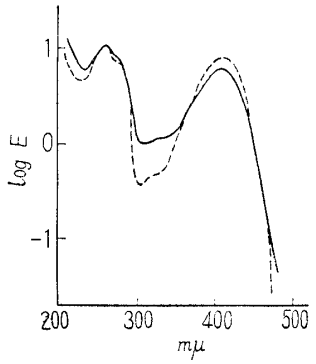

Fig. 1.

Comparison of Ultraviolet Absorption Spectra

—— Green fluorescent substance

------ 6,7-Dimethylribolumazine

from its Rf value and UV spectrum. Judging from the above results, it was found that 4-ribitylamino-5-aminouracil salt hardly reacts with acetoin when they were incubated at 37° for 3 hr., the reaction between them occurring only in the presence of an enzyme to produce 6,7-dimethylriboluma-zine and further riboflavin.

Discussion

It is believed that the biosynthesis of riboflavin is effected through a pteridine compound from the fact[2,3] that riboflavin (IV) is readily produced chemically[1] or by the action of enzyme from 6,7-dimethylribolumazine (III), which is found in the mycelium of *Er. ashbyii*.

Based on this idea, the reaction between 4-ribitylamino-5-aminouracil (II), which is regarded as a precursor of (III), and the crude enzyme produced from the yellow or leuco strain of *Er. ashbyii* was attempted, and the formation of (III) and (IV) was confirmed. In this case, however, if the reactants contained no acetoin, increase of riboflavin was not observed even in the presence of acetic acid, pyruvic acid, acetaldehyde, or formic acid, and therefore presence of acetoin was necessary in this reaction.

As reported previously,[1] diacetyl was found neither in the mycelium nor in the culture medium, but acetoin was found in both in 150 γ/g. and 40 mg./L., respectively. Hence, it seems that as soon as (II) is produced in the mycelium, it reacts with acetoin to form riboflavin through (III). Although free 4-ribitylamino-5-aminouracil is unstable, the aqueous solution of its salt is highly reactive and instantly combines with diacetyl at room temperature to produce (III) even in the absence of enzyme. Therefore, if diacetyl is added instead of acetoin in the reaction, (III) is first produced chemically, which is, however, converted to riboflavin (IV) by the action of enzyme.

Unlike diacetyl, acetoin is weak in reactivity and so it reacts with (II) only on heating. The product is evidently 6,7-dimethylribolumazine from its m.p., Rf value, and IR spectrum, and from the fact that it produces (IV) by the action of enzyme. Although acetoin hardly reacts with (II) at room temperature or at 37°, it produces (III) under such mild conditions if enzyme coexists. It is not yet clear whether the formation of (III) from (II) in the mycelium is effected by the direct combination of acetoin and subsequent dehydrogenation or by the oxidation of acetoin to diacetyl and subsequent participation of the product in the reaction.

When the reaction in which (III) is formed from (II) and diacetyl is compared with that in which (IV) is formed from (III) and diacetyl, the first is assumed to be chemically easier because it is a reaction between active *o*-diamino groups and *o*-diketone, and in fact it proceeded very smoothly. As reported previously,[1] the reaction of (III) with diacetyl sets in only on heating at 120~130° to produce riboflavin chemically, but the reaction between (III) and acetoin does not occur under the same conditions, it proceeding only in the presence of a small quantity of piperidine. Compared with the above reaction, the reaction in which (III) is formed from (II) proceeds very smoothly at room temperature when diacetyl is present, or on heating in the presence of acetoin. On the other hand, comparison of the above two reactions in the presence of enzyme showed that (IV) was produced from the uracil compound (II) via (III) in as poor a yield as 1.4%, while the formation of (IV) from (III) was very easy and the yield was abont 20%.

As mentioned above, acetoin is necessary for the formation of (III) from (II), and this is very interesting when compared with the experiment of Goodwin *et al.*,[4] in which acetylmethylcarbinol(2-^{14}C) (acetoin) was added to the medium during the cultivation of *Er. ashbyii* and the resulting riboflavin examined by degradation, one-half of the radioactivity was incorporated in the methyl group attached to the A-ring of the isoalloxazine

4) T. W. Goodwinn, D. H. Treble : Biochem. J., **70**, 14(1958).

ring, and the remaining radioactivity was confined only to 5-C and 8-C. In short, acetoin is a direct material for the biosynthesis of the pteridine ring and seems to be related to the formation of the benzene ring in riboflavin.

Summary

4-Ribitylamino-5-aminouracil (II), which is regarded as an intermediate in the biosynthesis of riboflavin, was synthesized and the formation of 6,7-dimethylribolumazine (III) and riboflavin (IV) was examined in three cases, in which a mixture of (II) and a crude enzyme solution prepared from *Er. ashbyii*, a mixture of (II) and acetoin, and a mixture of (II), acetoin, and a crude enzyme solution were each incubated at 37°. In the first two cases, the formation of (III) and (IV) was not observed, but in the last case the formation of a green fluorescent substance and increase of (IV) were recognized. The green fluorescent substance was confirmed to be (III) from its Rf values and ultraviolet spectrum.

From these results, it seems reasonable to think that riboflavin which is an isoalloxazine derivative is biosynthesized in the mycelium of *Er. ashbyii* from a pyrimidine compound (II) through a pteridine compound (III).

(Received April 22, 1959)

UDC 549.92.07

96. **Kyosuke Tsuda, Nobuo Ikekawa, und Shigeo Nozoe**: Untersuchungen über Steroide. XIII.[1] Mattox-Umlagerung von Reichsteins Substanz S bei der Ketalisierung mit 2-Methyl-2-äthyl-1,3-dioxolan.

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Die Bildung eines Nebenprodukts bei der 3,20-Bis-ketalisierungsreaktion von Reichsteins Substanz S (I) mittels Äthylenglykol in Benzol in Gegenwart von *p*-Toluolsulfonsäure als Katalysator war erstmalig von Antonucci, *et al.*[2] beschrieben worden, obwohl dessen Charakterisierung noch nicht ausgeführt worden ist. Vor kurzem berichteten Evans, *et al.*,[3] dass beim Ketalisieren von 4,5α-Dihydrocortison (17α,21-Dihydroxyallopregnan-3,11,20-trion) unter analogen Bedingungen neben 3,20-Bis-äthylenketal und 3-Monoäthylenketal noch eine Verbindung der Formel $C_{27}H_{40}O_7$ gebildet wurde. Hierbei wurde bewiesen, dass dieses Nebenprodukt eine 3-Äthylenketal- und eine 11-Oxo-Gruppe auf seinem Steroid-Gerüste besitzt, aber keine OH-Gruppe in der Seitenkette. Damit vermuteten diese Autoren, dass die Dihydroxyaceton-Gruppierung an C-17 des 4,5α-Dihydrocortisons unter den Angriff der zwei Mols Äthylenglykols zur Spiroketal- oder Tetraoxadekalin-Gruppierung verwandelt würde und keine OH-Gruppe sich in der Seitenkettemehr befände. Jedenfalls wurde die Ausbeute der normalen Ketal-Verbindungen bei dieser Reaktionen durch Hervorkommen der Nebenprodukte zurückgehalten.

Wir haben nun auch das Reichsteins Substanz S (I) unter etwas modifizierten Bedingungen ketalisiert. Als Ketalisierungsmittel diente 2-Methyl-2-äthyl-1,3-dioxolan (Methyläthylketon-äthylenketal).[4] Die Reaktion wurde in Dioxan in Gegenwart von *p*-Toluolsul-

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