tained underwent smooth oxidation by alkaline potassium ferricyanide to furnish the 2(1*H*)-pyridones, from which 4-acetyl and 4-formyl compounds were recovered through mild acid hydrolysis, and were then reduced according to Huang-Minlon, thus yielding 4-ethyl- and 4-methyl-2(1*H*)-pyridones in good yields. Yields were good throughout the entire series of reactions.

(Received June 5, 1958)

UDC: *577.164.12*: *545.84*

122. Satoru Kuwada, Toru Masuda, Toyokazu Kishi, and Mitsuko Asai:

Application of Chromatography. XXXVI.* Biosynthesis of Riboflavin. (2).** Riboflavin-synthesizing Enzyme extracted from *Eremothecium ashbyii*.

(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.****)

The assumption that production of riboflavin by *Er. ashbyii* probably goes through 6,7-dimethylribolumazine came from the fact that one of the authors (T.M.) once prepared riboflavin by the *in vitro* reaction of this lumazine derivative with diacetyl or acetoin. Katagiri, *et al.*[1] thereafter confirmed the formation of riboflavin from the 6,7-dimethylribolumazine sent from the authors, using a cell-free extract of *E. coli* and resting cells of the acetone·butanol-producing microörganism (*Clostridium acetobutyricum*), as well as a solution of the crude enzyme extracted from *Er. ashbyii*, confirming the assumption to a certain extent. They reported, however, that while addition of pyruvic acid in their reaction increased the formation of riboflavin, addition of diacetyl did not cause such a phenomenon. Of course, it is natural to think that a reaction *in vitro* does not necessarily take the same course *in vivo*, but as the components of the crude enzyme of *Er. ashbyii* were considered to be varied depending on the method of preparing the enzyme, attempts were made to reëxamine the result of Katagiri, *et al.*, and some new findings were obtained which are presented in this report.

Experimental

1) **Preparation of a Solution of the Crude Enzyme of *Er. ashbyii*—*Er. ashbyii*** was cultivated in the peptone medium described in the previous paper* for 64 hrs. with shaking and the filtered mycelium was lyophilized. Five grams of the lyophilized mycelium was thawed in a mortar and ground with 7 g. of alumina (W-800, commercial preparation of Wako Pure Chemicals, Ltd.) and 20 cc. of Sörensen-Palitzsch's phosphate buffer (pH 7.0) for 15~30 mins. under ice-cooling. The mixture thus prepared was centrifuged for 30 mins., at $6.7\times10^3 g$, in the International Refrigerated Centrifuge, the supernatant was dialyzed in a cellophane tube against phosphate buffer (pH 7.0) in the cold, and finally the content of the tube was diluted to 50 cc. with the same buffer solution. The crude enzyme solution was freshly prepared in this manner immediately before each experiment and the riboflavin contained in each lot was determined to obtain the blank test value for subsequent experiments. The content of the whole protein in the solution, as determined by the Folin's method, was ca. 300 γ/cc. when calculated as N (serum albumin was used as the standard).

2) **Effect of the Crude Enzyme Solution on 6,7-Dimethylribolumazine**—A 0.2-cc. portion of a $2\times10^{-3} M$ aqueous solution of 6,7-dimethylribolumazine was placed in a brown micro-test tube, 0.2 cc. of the above-mentioned crude enzyme solution was added, and incubated at 37° for 1 hr. The tube was immersed in a boiling water for 10 mins. to interrupt the reaction and the content was applied in a straight line on Toyo Roshi No. 5B filter paper, 47×40 cm., and developed with EtOH·BuOH·H_2O

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* Part XXXV: This Bulletin, **6**, 523(1958).

** "Biosynthesis of Riboflavin by *Er. ashbyii*" (This Bulletin, **5**, 136(1957)) by T. Masuda is considered as Part (1) of this work.

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1) H. Katagiri, *et al.*: Vitamins (Japan), **12**, 480(1957); **14**, 164, 695, 702(1958).

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(15:50:35) in a dark room. Of the yellow, green, and purple fluorescent spots detected under ultraviolet light, the yellow fluorescent spot corresponding to Rf≒0.3 was cut out, extracted with 5 cc. of hot water, and the ultraviolet spectrum of the extract was measured to give the curve shown in Fig. 1. The curve is in good accord with that of riboflavin, E at 450 mμ being 0.084, and the quantity of

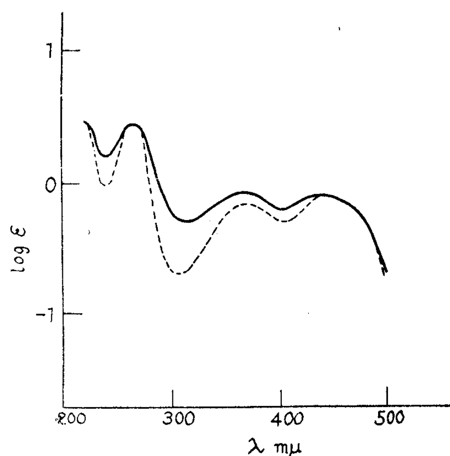

Fig. 1. U.V.-Absorption Spectra of Yellow Fluorescent Substance and Riboflavin

(Remark: U.V. absorption spectrum of riboflavin in the crude enzyme solution is almost impossible to determine.)

—— Yellow fluorescent substance
---- Riboflavin

riboflavin calculated from the value is $3.5\times10^{-8}M$. When developed with AcOH•BuOH•H_2O (1:4:5) and pyridine•BuOH•H_2O (3:4:7), the sample gave yellow fluorescent spots having Rf 0.29 and 0.60, respectively, and these Rf values also coincide with the corresponding values of riboflavin. By paper ionophoresis of the sample in Theorell buffer (pH 7.0, μ=0.1) at 400 V for 3 hrs., it migrated by 1.0 cm. toward the anode. This, too, is quite the same as the migration of riboflavin.

Next, 0.1 cc. of a $2\times10^{-3}M$ aqueous solution of 6,7-dimethylribolumazine was reacted with 0.1 cc. of the crude enzyme solution as above, and the whole reaction mixture was applied on two strips of Toyo Roshi No. 5B filter papar, 3×45 cm., and developed with the before-mentioned EtOH•BuOH•H_2O solvent system. The fluorescent spots corresponding to riboflavin were cut out, extracted with 5 cc. of hot water for 30 mins., and riboflavin in the extract was determined by bioassay using *L. casei*. The average value of five experiments was 4.4 γ.

For the blank test 0.1 cc. of the crude enzyme solution was immersed in a boiling water for 10 mins. and treated in the same way as above to determine the amount of riboflavin in the solution. The average value of five blank tests was 1.15 γ. Since $2\times10^{-7}M$ of 6,7-dimethylribolumazine was used as the starting material and since the lumazine compound is to produce equimolecule of riboflavin in theory, the yield of the resulting riboflavin is $8.1\times10^{-9}\times100/2\times10^{-7}$=4.6% of the theoretical.

3) Relationship between the Time of Action of the Crude Enzyme Solution and Quantity of Riboflavin produced—A mixture of 0.1 cc. (13.15 γ) of a $4\times10^{-4}M$ aqueous solution of 6,7-dimethylribolumazine and 0.1 cc. of the crude enzyme solution was incubated in a micro-test tube at 37° for various length of time as shown in Table I. The whole of each reaction mixture, after immersion in boiling water for 10 mins., was developed on a paper strip as in 2) and the fluorescent spot corresponding to riboflavin was cut out and extracted with 3 cc. of a 2% warm aqueous solution of saccharin for 30 mins. One cc. each of 10% AcOH and H_2O were added to the extract and the intensity of fluorescence of the

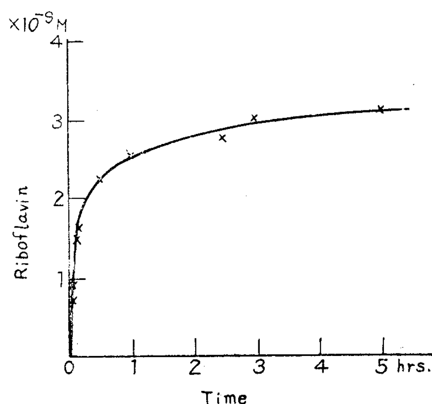

Fig. 2. Relationship between the Time of Action of the Crude Enzyme Solution and the Quantity of Riboflavin produced

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mixture was measured with the Kotaki fluorometer to determine the riboflavin therein. A paper strip of the same size and the same quality was treated as above for the blank test. Also, for determination of riboflavin in the crude enzyme solution, 0.1 cc. of the solution was immersed in boiling water and then processed under the same conditions as above. On the other hand, the standard value of riboflavin was obtained by subjecting 1 γ of riboflavin to paper partition chromatography and working up in the same manner as above. Fig. 2 is a graph representing this result.

4) Relationship between the Quantity of Crude Enzyme Solution added and the Quantity of Riboflavin produced—A 0.1-cc. portion of the $2\times10^{-3}M$ aqueous solution of 6,7-dimethylribolumazine was mixed with the crude enzyme solution in various ratios and incubated for 30 mins. The whole of each reaction mixture was developed as before and the spot corresponding to riboflavin was cut out and extracted with 5 cc. of a warm 2% saccharin solution for 30 mins. Two cc. of the extract was mixed with 2 cc. of $2N$ NaOH and photodecomposed in a test tube by irradiation for 1 hr. with a 20-W fluorescent lamp from a 10-cm. distance. The decomposition product was shaken with 0.6 cc. of glacial AcOH and 8 cc. of $CHCl_3$, the $CHCl_3$ layer was separated, and dried over anhyd. Na_2SO_4. The intensity of fluorescence of the solution was measured by the Kotaki fluorometer to give the result shown in Fig. 3.

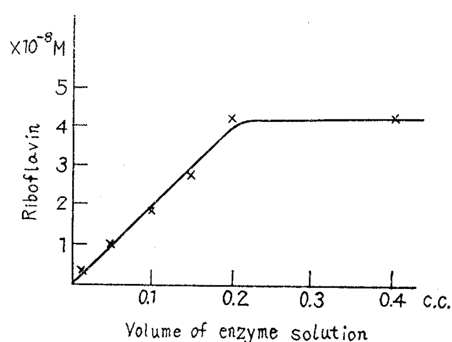

Fig. 3. Relationship between the Quantity of the Crude Enzyme Solution added and the Quantity of Riboflavin produced

In this case, the fluorescent solution obtained by treating 1.0 and 0.5 γ of riboflavin in the same manner as above was used as a standard.

5) Relationship between Concentration of the Substrate and the Quantity of Riboflavin produced—A 0.1-cc. portion of the crude enzyme solution was mixed with 6,7-dimethylribolumazine in various ratios and the mixture was processed in the same way as in 4) to give the results shown in Fig. 4.

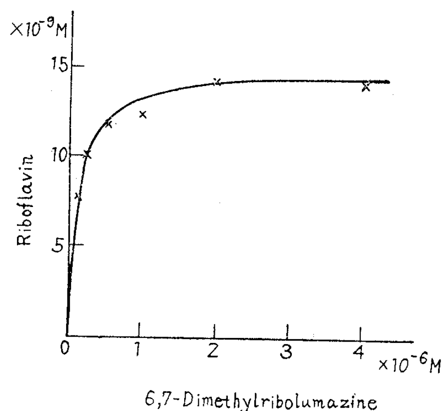

Fig. 4. Relationship between Concentration of the Substrate and Quantity of Riboflavin produced

6) Optimum pH and Temperature for the Action of the Crude Enzyme Solution—Examinations were made to find the pH and temperature at which the crude enzyme solution displays its maximum activity.

A mixture of 1.0 cc. of phosphate buffer (pH 4.8~8.8 as shown in Table I), 0.2 cc. of the crude enzyme solution, and 0.2 cc. of 6,7-dimethylribolumazine solution ($4\times10^{-3}M$) was incubated at 37° for 30 mins. The reaction mixture was dipped in boiling water for a while and evaporated to 1.0 cc., and 0.2 cc. of the concentrate was subjected to paper partition chromatography to separate into each component. The portion of the chromatogram corresponding to riboflavin was cut out and the amount of riboflavin therein was determined. The result is shown in Table I, which indicates that the activity of the crude enzyme solution is maximum at pH 7.0 and suddenly decreases in alkaline pH.

A mixture of 0.1 cc. of the same crude enzyme solution and 0.1 cc. of 6,7-dimethylribolumazine solution ($4\times10^{-3}M$) was reacted for 30 mins. at 10~70°, as shown in Table II, and the resulting riboflavin was determined. The result showed that the formation of riboflavin was maximum at around 45°.

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TABLE I. Optimum pH for the Action of the Crude Enzyme Solution

Test No.	pH of phosphate buffer	Riboflavin ($\times 10^{-9}M$)	
		Quantity detected	Quantity produced
1	4.8	9.4	6.0
2	5.7	10.2	6.8
3	6.3	12.4	9.0
4	6.5	13.3	9.9
5	7.0	15.3	11.9
6	7.4	11.8	8.4
7	8.0	7.1	3.7
8	8.8	4.3	0.9
9*	7.0	3.4	

* Blank test value of 0.2 cc. of the crude enzyme solution treated with heat.

TABLE II. Optimum Temperature for the Action of the Crude Enzyme Solution

Test No.	Temp. of action (°C)	Riboflavin ($\times 10^{-9}M$)	
		Quantity detected	Quantity produced
1	10	29.1	1.7
2	20	31.3	3.9
3	25	36.0	8.6
4	30	39.6	12.2
5	35	47.9	20.5
6	37	48.9	21.5
7	40	52.6	25.2
8	42	53.2	25.8
9	45	59.7	32.3
10	50	57.4	30.0
11	55	56.5	29.1
12	60	51.0	23.6
13	70	31.6	4.2
14*		27.4	

* Blank test value of 0.1 cc. of the crude enzyme solution treated with heat.

7) **Effect of the Crude Enzyme Solution on 6-Methyl-7-hydroxyribolumazine**—A mixture of 0.1 cc. of aqueous solution ($3.34 \times 10^{-4}M$) of 6-methyl-7-hydroxyribolumazine and 0.1 cc. of the crude enzyme solution was incubated in a brown test tube at 37° for 1 hr. and the reaction mixture was subjected to paper partition chromatography. The spots corresponding to 6-methyl-7-hydroxyribolumazine and riboflavin were cut out and each extracted with 5 cc. of warm water for 30 mins. The intensity of fluorescence of each extract was measured with the Kotaki fluorometer to determine the component. The standard solution for this test was prepared by treating the above 6-methyl-7-hydroxyribolumazine solution alone in the same way as above. In parallel with this test, the same sample was migrated on paper in Theorell buffer (pH 7.0, μ=0.1) at 300 V for 3 hrs. and the two separated fluorescent spots were treated as above. The results from both tests are presented in Table III. No. 2 in the table shows the value obtained by heating the crude enzyme solution for 10 mins. and then reacting with 6-methyl-7-hydroxyribolumazine.

TABLE III. Effect of the Crude Enzyme Solution on 6-Methyl-7-hydroxyribolumazine

Test No.	Quantity of 6-methyl-7-hydroxyribolumazine detected ($\times 10^{-8}M$)		Quantity of riboflavin detected ($\times 10^{-9}M$)	
	Separation by paper partition chromatography	Separation by paper ionophoresis	Separation by paper partition chromatography	Separation by paper ionophoresis
1	3.00	3.27	3.59	3.51
2	3.27	3.34	3.75	3.67

As 6-methyl-7-hydroxyribolumazine is an acid substance, its 3% aqueous solution showing pH 2.4, it may inhibit activity of the crude enzyme solution. To make this point clear, the sample (65.6 γ/0.1 cc. $=2 \times 10^{-7}M$) was dissolved in a phosphate buffer and the solution, after being adjusted to pH 6.8, was

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incubated and treated as before. As is evident from the results shown in Tables III and IV, 6-methyl-7-hydroxyribolumazine is not affected by the crude enzyme solution and is therefore not converted into riboflavin.

TABLE IV. Effect of the Crude Enzyme Solution on 6-Methyl-7-hydroxyribolumazine

Test No.	Quantity of 6-methyl-7-hydroxyribolumazine detected ($\times 10^{-7}M$)		Quantity of riboflavin detected ($\times 10^{-10}M$)
	Separation by paper partition chromatography	Separation by paper ionophoresis	
1	1.90		7.57
2	1.89		6.88
3	1.91		8.95
4		1.96	
5	1.90		9.10

No. 5 is the control value obtained by treating the enzyme solution with heat and subjecting to the same experiment.

8) The Purple-fluorescent Substance produced by the Action of the Crude Enzyme Solution on 6,7-Dimethylribolumazine—A mixture of 6,7-dimethylribolumazine and the crude enzyme solution was incubated at 37° for more than 30 mins. according to the method described in 2), and the reaction mixture was subjected to paper partition chromatography. The purple fluorescent spot at Rf 0.23 was cut out and extracted with hot water. The extract exhibited the U.V. spectrum shown in Fig. 5, which closely resembles that of 6-methyl-7-hydroxyribolumazine. As seen from Table V, the Rf value in paper partition chromatography and migration distance in paper ionophoresis of +4.0 cm. (Theorell buffer, pH 7.0, μ=0.1, 400V, 3 hrs.) of this substance are also in complete agreement with those of 6-methyl-7-hydroxyribolumazine.

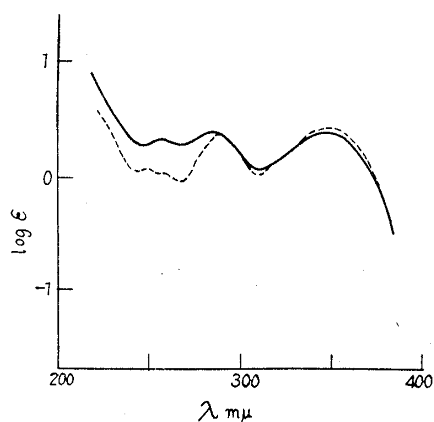

Fig. 5. Ultraviolet Absorption Spectra of Purple fluorescent Substance and 6-Methyl-7-hydroxyribolumazine

—— Purple-fluorescent substance
- - - - 6-Methyl-7-hydroxyribolumazine

TABLE V. Rf Values and Migration Distance of Purple-fluorescent Substance

Solvent system for development	Rf values	
	Purple-fluores. subst. in the reaction mixt.	6-Methyl-7-hydroxy-ribolumazine
$AcOH \cdot BuOH \cdot H_2O$ (1:4:5)	0.14	0.15
$EtOH \cdot BuOH \cdot H_2O$ (15:50:35)	0.23	0.23
$Pyridine \cdot BuOH \cdot H_2O$ (3:4:7)	0.44	0.43
Migration distance in paper ionophoresis	+4.0	+4.0

Discussion

For the purpose of reëxamining the result of Katagiri, *et al.*,[1] in which they confirmed the formation of riboflavin from 6,7-dimethylribolumazine by the action of an enzyme extracted from the mycelium of *Er. ashbyii*, the present authors also prepared a crude

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enzyme solution from the mycelium of the microörganism, and incubation of the crude enzyme solution with 6,7-dimethylribolumazine confirmed the production of riboflavin. Quantitative determination of the resulting riboflavin by the lumiflavin fluorescence method was found to give a value approximating that of bioassay.

The result of this experiment indicates that when the crude enzyme solution is reacted with 6,7-dimethylribolumazine under the conditions described in the experimental part, the quantity of riboflavin originally contained in the solution increases about 3 times. Studies are now under way on the carbon donor necessary to form the aromatic ring of the isoalloxazine ring.

The riboflavin-synthesizing enzyme used in the present work not only is a crude preparation but also seems to be a group consisting of various enzymes, and therefore, it is necessary to measure its activity. It would be better if the activity can be measured by a pure crystalline substrate with a definite structure such as 6,7-dimethylribolumazine. To make this point clear, various experiments were attempted. To begin with, when the crude enzyme solution was acted on the substrate, the formation of riboflavin was remarkable at the beginning but it decreased gradually after 1 hour. This seems to be attributed to inactivation of the enzyme, lowering of the concentration of the substrate, and inhibition of the reaction by the products formed. The relationship between amount of the crude enzyme solution and quantity of the resulting riboflavin was proportional up to a definite volume (0.2 cc. under the present conditions) of the enzyme solution, and that between concentration of the substrate and quantity of the resulting riboflavin was also rectilinear at lower concentrations of the former. Although it is not yet clear whether riboflavin was produced by a one-step reaction or not, the Michaelis constant of the enzyme, as measured by the Lineweaver's method, was $Km \fallingdotseq 3.3 \times 10^{-3}$.

Incubation of the crude enzyme solution with 6,7-dimethylribolumazine produced only riboflavin at the beginning, but after 30 mins. there was also formed a purple-fluorescent substance, which was found to be identical with 6-methyl-7-hydroxyribolumazine.

However, incubation of the crude enzyme solution with 6-methyl-7-hydroxyribolumazine neither produced riboflavin nor decreased the amount of 6-methyl-7-hydroxyribolumazine, and therefore it is untenable to think that the latter is an intermediate of riboflavin. Judging from the result of simultaneous determination of riboflavin, 6,7-dimethylribolumazine, and 6-methyl-7-hydroxyribolumazine in the fermentation product of *Er. ashbyii*, the authors previously supposed[2] that 6-methyl-7-hydroxyribolumazine is the final product in the metabolism and that it is probably produced by the reaction of probable intermediate, 4-ribitylamino-5-aminouracil, with pyruvic acid. From results of the present work, however, it became necessary to consider another route in which action of the enzyme of *Er. ashbyii* on 6,7-dimethylribolumazine produces both riboflavin and 6-methyl-7-hydroxyribolumazine at the same time as shown in Chart 1.

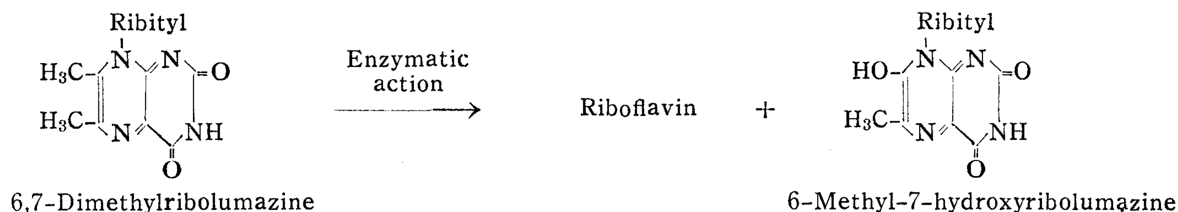

Chart 1.

It still remains unclarified, however, whether riboflavin and 6-methyl-7-hydroxyribolumazine are produced by the same enzyme or by different enzymes and how this seemingly abnormal reaction is effected.

2) T. Masuda, T. Kishi, M. Asai, S. Kuwada: This Bulletin, **6**, 523(1958).

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The authors wish to express their grateful appreciation to Mr. Yutaka Shiraishi for his cooperation in the fermentation of *Er. ashbyii* and to Dr. Minoru Goto for his performance of bioassay with *L. casei*. Thanks are also due to members of the biochemical section of this Laboratories for their valuable advices.

Summary

The crude enzyme solution, prepared by extracting the ground mycelium of *Er. ashbyii* with a phosphate buffer and dialyzing the extract against the same buffer solution, was incubated with 6,7-dimethylribolumazine at 37°. Paper partition chromatography of the reaction mixture gave a yellow and a purple fluorescent spot. The former spot (Rf 0.3; solvent-system: EtOH・BuOH・H_2O) was cut out and extracted with hot water, and the ultraviolet spectrum of the extract was measured, showing that it is identical with that of riboflavin. The riboflavin was determined by bioassay with *L. casei* and by various chemical methods such as the lumiflavin-fluorescence method and others.

Next, investigation was made on relationship between quantity of the resulting riboflavin and reaction time, quantity of the crude enzyme solution added, and concentration of the substrate, and further on the optimal pH and temperature for the action of the enzyme solution.

Lastly, the above purple-fluorescent spot (Rf 0.23; solvent-system: EtOH・BuOH・H_2O) was proved to be that of 6-methyl-7-hydroxyribolumazine, and a new route was proposed for the formation of this compound. Further, the fact that the lumazine derivative was not affected by the crude enzyme solution made more certain the authors' previous assumption[2] that the compound is a final product in the metabolism by *Er. ashbyii*.

(Received June 6, 1958)

UDC 547.787.1

123. M. Tomoeda: Studies in Stereochemistry. XX.* *dl*-Phenylserinols: A New Synthesis and its Stereochemical Findings. (7).[1] An Isomerization of *dl-threo*-2-Phenyl-4-α-hydroxybenzyl-2-oxazoline to *dl-threo*-4-Hydroxymethyl-2,5-diphenyl-2-oxazoline in Basic Media: The Correction of the Previously Proposed Structure for the Isomerization Product.

(*Pharmaceutical Institute, Medical Faculty, University of Kyushu***)

In the previous papers of this series,[2,3] it has been reported that boiling of *dl-threo*-1-phenyl-2-amino-3-bromopropyl benzoate (I) in benzene yielded nitrogen-containing colorless needles (A) in accompaniment with *dl-threo*-2-phenyl-4-α-hydroxybenzyl-2-oxazoline (II). The latter isomerized into (A) in the presence of alkali, suggesting (II) to be a precursor of (A). Furthermore, the structure of (A) has been proposed as *dl*-2-benzamido-3-phenyl-2-propen-1-ol (IIIa) or *dl*-2-benzoylimino-3-phenylpropanol (IIIb) from the result of microanalysis, molecular weight determination, infrared and ultraviolet spectral analyses, and also by the fact that on hydrogenation, (A) absorbed hydrogen to give *dl*-2-benzamido-3-phenylpropanol (IV).

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* This constitutes a part of a series entitled "Studies in Stereochemistry" by T. Taguchi.
** Katakasu, Fukuoka (友枝宗光).
1) Part (6): This Bulletin, **5**, 335(1957).
2) Part (2): *Ibid.*, **4**, 80(1956).
3) Part (3): *Ibid.*, **4**, 473(1956).

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