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Summary

dl-Lyxose and *dl*-xylose were synthesized by the *cis*-hydroxylation of *dl-trans*-1,1-diethoxy-5-tetrahydropyran-2-yloxypent-3-en-2-ol (XII) and it was anticipated that they would also be formed by the *trans*-hydroxylation of *dl-cis*-1,1-diethoxy-5-tetrahydropyran-2-yloxypent-3-en-2-ol (IV).

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78. Mitsuko Asai, Toru Masuda, and Satoru Kuwada : Application of Chromatography. XLII.*1 Formation of Riboflavin by Enzyme System from Leuco-type Strain of *Eremothecium ashbyii*.

(*Research Laboratories, Takeda Chemical Industries, Ltd.**2)

The mechanism[1] for synthesis of riboflavin by *Eremothecium ashbyii* presumed by the authors was further investigated biochemically by Katagiri, *et al.*[2] and the present authors,[3] and it was found that 6,7-dimethylribolumazine is an intermediate in the biosynthesis of riboflavin. Korte, *et al.*[4] and Maley, *et al.*[5] also duplicated the experiment using ^{14}C-labeled 6,7-dimethylribolumazine and confirmed the above result.

It was later reported[6] that the action of crude enzyme solutions prepared from yellow-type and leuco-type strain of *Er. ashbyii* on 4-ribitylamino-5-aminouracil and acetoin produced both 6,7-dimethylribolumazine and riboflavin, and Katagiri, *et al.*[7] also recognized the result using an enzyme solution prepared from yellow-type *Er. ashbyii*.

The above-mentioned leuco-type strain was produced in the course of successive cultivation of the yellow-type strain at the Fermentation Institute, Osaka, and it yielded only 200 γ/g. (wet mycelium) of riboflavin after 88 hours of culture, whereas the ordinary yellow-type strain produces about 8,800 γ/g. after 63 hours of culture, but no remarkable difference was found between the two strains in the amount of the mycelium produced.[8]

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*1 Part XLI : This Bulletin, **8**, 798 (1960).

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

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Recently, a leuco-type strain of *Er. ashbyii* was received from Prof. Takata of the Department of Technology, Kyoto University. He obtained the strain from Prof. Naganishi of the Hiroshima University and cultivated successively for a long time, but it did not revert to the yellow-type. This strain was cultivated on the same peptone medium[9] as that used for the yellow-type strain and about 20 g./L. (broth) and 24 g./L. of the mycelium were obtained after 70 and 120 hours, respectively. However, when 0.8% meat extract was substituted by 0.5% corn-steep liquor, the yield of the mycelium was raised to 34.5 g./L. after 65 hours and 40.5 g./L. after 94 hours.*3 In the present experiments, the mycelium obtained by the latter method was employed.

The mycelium filtered from the broth was extracted with a definite amount of water and the filtered broth was concentrated to one-tenth the original volume. The sample solutions thus obtained were applied on filter paper, developed with a solvent system containing butanol and ethanol, and the chromatograms were observed under ultraviolet ray of 2537 Å. In this case, a faint yellow spot of riboflavin and some unidentified light-absorbing spots were detected, but no spots of lumazine-series substances were observed. The chromatograms were then sprayed with the Ehrlich reagent, when tryptophan was detected but no spot of *l*-3-hydroxykynurenine, which is present in the yellow-type strain, was observed (see Table I).

Since the metabolism of amino acids in the leuco-type strain seemed to be different from that of amino acids in the yellow-type strain, two extracts prepared from this strain and the yellow-type strain were subjected to two-dimensional chromatography and the amino acids detected by the Ninhydrin method were compared, giving the results shown in Table II. As is evident from the table, hydroxykynurenine was not observed on the chromatogram from the leuco-type strain, and, in its stead, proline which is absent in the yellow-type strain was detected. The meaning of the above metabolism of amino acids arouses a considerable interest.

The yellow fluorescent spot of riboflavin mentioned above was cut out and extracted with 2% saccharine solution. The extract was subjected to photodecomposition and riboflavin in it was determined, finding about 3 γ/g. (wet mycelium). As is obvious from this result, production of riboflavin by the leuco-type strain given by Prof. Takata was low compared to the leuco-type strain stored in the Fermentation Institute and the metabolism of amino acids was also a little different. It was therefore anticipated that the enzyme system of the former strain also might show a different activity.

Consequently, it became necessary to investigate whether this leuco-type strain has an enzyme system having the ability to produce riboflavin from 6,7-dimethylribolumazine or from 4-ribitylamino-5-aminouracil and acetoin, as once observed with the yellow-type strain, and some experiments were carried out.

A crude enzyme solution was prepared as in the previous report[3] from the mycelium of the leuco-type strain obtained by shaking culture for 65~94 hours, a part of the solution was used directly for enzymatic reactions, and the rest was freeze-dried, stored in a cool place, and used for the same purpose as a solution in a cold *M*/15 phosphate buffer (pH 7.0), when required. 6,7-Dimethylribolumazine solution was added to the crude enzyme solution, the mixture was incubated at 37° for 3 hours, and the reaction mixture, after heating for 10 minutes, was submitted to paper partition chromatography. A yellow fluorescent spot (1) with Rf 0.35, a purple fluorescent spot (2) with Rf 0.22, and a green fluorescent spot (3) with Rf 0.20 were detected on the chromatogram. The spot (1) was cut out and extracted with water and, from ultraviolet spectrum of the extract, the spot was

9) T. Masuda, T. Kishi, M. Asai, S. Kuwada : *Ibid.*, **6**, 523 (1958).

*3 Increase of the mycelium by use of corn-steep liquor was suggested by Prof. Takata (Vitamins (Kyoto), **21**, 168 (1960)).

confirmed to be of riboflavin. It is evident from their ultraviolet spectra that the spots (2) and (3) are the unchanged substrate and 6-methyl-7-hydroxyribolumazine, respectively. As control, (a) the enzyme solution treated with heat and (b) the enzyme solution mixed with the substrate solution were treated in the same manner as above. In this case, no fluorescent spot was found in (a) and only the spot of the substrate was detected in (b).

Next, 6,7-dimethylribolumazine solution ($1.25\times10^{-3}M$) was added to the crude enzyme solutions prepared from the leuco-type and yellow-type strains, the mixtures were treated as before, and riboflavin in their spots was determined to find its percentage against the substrate. The control was the same enzyme solution, heated and processed as above. The protein nitrogen in the enzyme solutions was determined by the Folin method to find their specific activity and it was found that the value of the enzyme solution from the yellow-type strain was a little higher than that of the enzyme solution from the leuco-type strain (see Table IV).

A similar experiment was carried out with 4-ribitylamino-5-aminouracil as a substrate and acetoin as a carbon donner. The chromatograms and amount of the resulting riboflavin obtained by the experiments with the enzyme solutions from the leuco-type and yellow-type strains are shown in Table V, and the quantity of riboflavin determined once more and the specific activity of the enzyme solutions are given in Table VI. As is evident from these tables, while the enzyme solution from the yellow-type strain produced riboflavin and 6-methyl-7-hydroxyribolumazine through 6,7-dimethylribolumazine from 4-ribitylamino-5-aminouracil and acetoin, the enzyme solution from the leuco-type strain did not yield these compounds at all.

From the experimental data mentioned above, it is assumed that the enzyme system of the leuco-type strain of *Er. ashbyii* is a little different from that of the yellow-type strain in properties such as the activity to metabolize amino acids. Further, though the former enzyme system has the ability to produce riboflavin from 6,7-dimethylribolumazine, it lacks the activity to yield riboflavin from 4-ribitylamino-5-aminouracil which is assumed to be a precursor of the lumazine compounds, through 6,7-dimethylribolumazine.

Experimental

1) Paper Partition Chromatography of the Extract of Leuco-type *Er. ashbyii* ——a) The leuco-type strain was cultivated on a peptone medium*4 for 72 hr. by the same conditions as in the case of the yellow-type strain, when the mycelium increased to 20.4 g. (wet)/L. (medium). After 120-hr. culture, however, the mycelium did not increase so much, amounting only to 24.3 g./L. Use of

Table I. Chromatograms of Mycelium Extract of Leuco-type *Er. ashbyii* and Concentrated Filtered Broth

Sample	Detection method	Rf value								
Mycelium extract	UV	0.02 ab(卄)	0.09 ab(+)	0.13 f (±)	0.35 Y (±)	0.37 ab(+)	0.38 B (±)	0.40 ab(+)	0.45 ab(+)	0.59 ab(卄)
	Ehrlich reagent						0.38 P			
Filtered broth	UV	0.04～0.09 BW(卄)			0.35 Y (±)	0.37 ab(+)	0.38 B (±)	0.40 ab(+)		0.52 VB(+)
	Ehrlich reagent	0.04～0.09 O								

Observation under ultraviolet ray : ab; light-absorbing spot, f; fluorescence, Y; yellow fluorescence, B; blue fluorescence, BW; bluish white fluorescence, VB; violet-blue fluorescence.
Coloration with Ehrlich reagent : P; purple, O; orange. (±)～(卄) intensity of fluorescence and absorption. Rf 0.35 Y; spot of ribofiavin, 0.38 P; spot of tryptophan.

*4 See reference (8); 0.8% polypeptone, 2.0% glucose, 0.8% meat extract, 0.1% $MgSO_4\cdot7H_2O$, 0.2% KH_2PO_4, 0.1% NaCl.

0.5% of corn-steep liquor instead of meat extract increased the mycelium to 34.5 g./L. after 65 hr. and to 40.5 g./L. after 94 hr. Three g. of the filtered mycelium was extracted with 2 cc. of H_2O at 80° and the filtered broth was concentrated to 1/10 the original volume to make a sample solution.

The sample solutions were developed on Toyo Roshi No, 5B filter paper with EtOH-BuOH-H_2O (15:50:35) and the chromatograms were observed under ultraviolet rays of 2537 Å. At the same time they were sprayed with the Ehrlich reagent to detect spots positive to the reagent. The results are shown in Table I.

b) The mycelium extract mentioned above and an extract prepared for comparison from the yellow-type mycelium were subjected to two-dimensional chromatography and the chromatograms were sprayed with a Ninhydrin solution to detect amino acids. The first developing solvent was *tert*-BuOH-MeCOEt-H_2O (4:4:2) and the second one was *tert*-BuOH-MeOH-H_2O (4:5:1). The results are summarized in Thble II. It is noted from this table, that the extract from the leuco-type mycelium does not contain hydroxykynurenine, which is present in the extract from the yellow-type mycelium, and instead it contains proline which is absent in the latter extract.

Table II. Comparison of Amino Acids in Leuco-type Strains

Leuco-type					Yellow-type				
Spot No.	Intensity	Rf First	Rf Second	Presumed amino acid	Spot No.	Intensity	Rf First	Rf Second	Presumed amino acid
1	++	0.00	0.00	Peptide	1	++	0.00	0.00	Peptide
2	+++	0.026	0.06	Lysine, histidine*	2	+++	0.026	0.08	Lysine, histidine*
3	+++	0.04	0.13	Aspartic acid, Glutamic acid*	3	+	0.065	0.11	Aspartic acid, Glutamic acid*
4	++	0.08	0.14	Glycine, serine*	4	++	0.09	0.16	Glycine, serine*
5	++	0.10	0.21	Threonine	5	+	0.10	0.21	Threonine
6	++	0.13	0.27	Alanine	6	+++	0.13	0.29	Alanine
7	+	0.195	0.28	Proline	8	++	0.30	0.27	Tyrosine
8	+	0.30	0.27	Tyrosine	9	+	0.34	0.25	Tryptophan
9	+	0.35	0.23	Tryptophan	10	+	0.28	0.40	Valine
10	++	0.29	0.41	Valine	11	+	0.43	0.35	Phenylalanine
11	+	0.43	0.36	Phenylalanine	12	++	0.48	0.46	Leucine
12	+	0.48	0.47	Leucine	13	+	0.34	0.17	Hydroxykynurenine
					14	+	0.42	0.12	——

* Overlapped spots were separated by two dimensional chromatography, using AcOH-BuOH-H_2O (1:4:5) and PhOH-H_2O.

2) Determination of Riboflavin produced by Leuco-type *Er. ashbyii* ——Five g. of the wet mycelium obtained by the above-mentioned culture was extracted with 5 cc. of H_2O at 80° for 10 min., 0.2 cc. of the extract was repeatedly applied in a line on each end of the two pieces of filter paper (4×43 cm.), and developed with EtOH-BuOH-H_2O (15:50:35). The yellow-fluorescent spot at Rf 0.35 was cut out and extracted with 5 cc. of 2% saccharine solution at 80° for 20 min., and 2 cc. of the extract, after being made alkaline with 2 cc. of 2*N* NaOH, was irradiated with a 20-W fluorescent lamp from a distance of 10 cm. for 1 hr. The solution thus treated was shaken with 0.5 cc. of glacial AcOH and 8 cc. of $CHCl_3$ to transfer lumiflavin produced by the photodecomposition of riboflavin into the $CHCl_3$ layer and finally the fluorescence of lumiflavin was compared with that of lumiflavin produced from standard riboflavin. As a result, it was found that 3 γ/g. (wet mycelium) of riboflavin was produced after 72 hr. in the culture on a medium containing meat extract and 2.18 γ/g. and 2.81 γ/g., in the culture on a medium containing corn-steep liquor instead of meat extract after 65 and 94 hr., respectively.

3) Synthesis of Riboflavin by the Enzyme System of Leuco-type *Er. ashbyii* ——a) Preparation of a crude enzyme solution : A crude enzyme solution was prepared according to the method reported in the previous paper[3] from the leuco-type mycelium obtained by 65- or 94-hr. culture. Part of the solution was directly used for enzymatic reactions and the rest was freeze-dried, stored in a desiccator in a cool place, and used for the same purpose, as shown in Table IV, as a solution in *M*/15 phosphate buffer of pH 7.0. The control enzyme solution was prepared from the yellow-type mycelium obtained by shaking culture for 44 or 68 hr.

b) Products produced when 6,7-dimethylribolumazine was used as substrate, and determination of the resulting riboflavin : To 1 cc. of the enzyme solution from the leuco-type mycelium in a brown test tube 1 cc. of a 6,7-dimethylribolumazine solution ($1.25\times10^{-3}M$) was added and the mixture was

incubated at 37° for 3 hr. The reaction mixture was treated with heat for 10 min. and 1 cc. of it was applied in a line on a sheet of filter paper with 30 cm. width, developed with EtOH-BuOH-H_2O (15:50:35) and observed under ultraviolet ray to give the chromatogram shown in Table III. Of the spots, those corresponding to Rf 0.35 and Rf 0.22 were cut out, extracted with H_2O, and the ultraviolet spectra of the extracts were measured to confirm that the former was the spot of riboflavin and the latter was the spot of 6-methyl-7-hydroxyribolumazine. The green fluorescent spot at Rf 0.20 is of course the spot of the unchanged substrate.

As control test, (1) the crude enzyme solution was heated at 100° for 10 min. and 1 cc. of it was diluted with 1 cc. of H_2O, and (2) the enzyme solution was treated with heat as above and 1 cc. of it was mixed with 1 cc. of the substrate solution, and both sample solutions were processed as before and their chromatograms were observed under ultraviolet ray. As a result, no fluorescent spot was detected in (1) and only a green fluorescent spot at Rf 0.20 was found in (2).

Table III. Change of 6,7-Dimethylribolumazine by the Enzyme of Leuco-type *Er. ashbyii*

Sample	Rf value		
	0.20 G	0.22 V	0.35 Y
Enzyme solution plus 6,7-dimethylribolumazine			
Enzyme solution treated with heat	—	—	—
Enzyme solution treated with heat plus 6,7-dimethylribolumazine	0.20 G		

Table IV. Amount of Riboflavin produced with 6,7-Dimethylribolumazine as Substrate

	Strain	Culture period (hr.)	Crude enzyme	Protein-N γ/0.2 cc. (N)	Riboflavin detected (γ)	Riboflavin formed μmole/0.2 cc. (γ)	(L)	(%)*	Specific activity L/N × 1000
1)	Leuco-type	65	liquid	11.3	1.32 (R = 0.05) (mean value in 2 expt.)	1.32	0.0035	2.8	0.310
			〃 control		0				
2)	〃	94	liquid	11.8	1.08 (R = 0.10) (mean value in 2 expt.)	1.08	0.00288	2.3	0.244
			〃 control		0				
3)	〃	65	solution of dried powder, 16 mg./cc.	15.0	1.49 (R = 0.13) (mean value in 2 expt.)	1.49	0.00395	3.16	0.263
			〃 control		0				
4)	〃	65	solution of dried powder, 30.4 mg./cc.	28.4	1.89 (R = 0.10) (mean value in 3 expt.)	1.89	0.00502	4.02	0.176
			〃 control		0				
5)	〃	94	solution of dried powder, 13.8 mg./cc.	17.5	1.41 (R = 0.05) (mean value in 2 expt.)	1.41	0.00374	2.99	0.214
			〃 control		0				
6)	〃	94	solution of dried powder, 26.8 mg./cc.	34.0	1.58 (R = 0.11) (mean value in 2 expt.)	1.58	0.0042	3.40	0.123
			〃 control		0				
7)	Yellow-type	44	liquid	11.85	3.25 (R = 0) (mean value in 2 expt.)	1.55	0.00412	3.3	0.348
			〃 control		1.70 (R = 0) (mean value in 2 expt.)				
8)	〃	68	liquid	9.32	3.97 (R = 0.10) (mean value in 2 expt.)	2.60	0.00692	5.54	0.743
			〃 control		1.37 (R = 0) (mean value in 2 expt.)				
9)	〃	44	solution of dried powder, 25 mg./cc.	22.8	5.2 (R = 0) (mean value in 2 expt.)	1.73	0.00460	3.68	0.210
			〃 control		3.47 (R = 0.19) (mean value in 2 expt.)				
10)	〃	68	solution of dried powder, 25.4 mg./cc.	20.45	7.10 (R = 0.40) (mean value in 2 expt.)	4.16	0.0110	8.85	0.540
			〃 control		2.94 (R = 0.21) (mean value in 3 expt.)				

* Percentage against substrate.

To 0.1 cc. of the crude enzyme solution in a microtest tube 0.1 cc. of the substrate solution was added and the mixture, after being incubated at 37° for 3 hr. and heated, was repeatedly applied on two pieces of filter paper (4×43 cm.) and developed with the solvent mentioned before. The yellow fluorescent spots at Rf 0.35 were cut out and extracted with 5 cc. of a 2% saccharine solution, and riboflavin in the extract was determined by the lumiflavin method. As a control test, 0.1 cc. of the crude enzyme solution treated with heat was mixed with 0.1 cc. of the substrate solution and the mixture was treated in the same manner as above. The protein-N of the enzyme solution was determined by the Folin method. The same experiment was also carried out on the crude enzyme solution obtained from the yellow-type mycelium and the result was compared with that obtained on the enzyme solution from the leuco-type mycelium (Table IV). As seen in the table, the enzyme solution from leuco-type mycelium also had the ability to produce riboflavin from 6,7-dimethylribolumazine, but seems to be a little weaker in the ability than that from the yellow-type mycelium.

c) Products when 4-ribitylamino-5-aminouracil was used as substrate and determination of the resulting riboflavin : In each of 2 test tubes, 1 cc. of the crude enzyme solution from the leuco-type mycelium was placed and one of them was heated and used as a control. To each of the test tubes, 1 mg. of 4-ribitylamino-5-aminouracil (3×10^{-6} mole.) and 5 mg. of acetoin were added and the mixtures were incubated at 37° for 3 hr. A 0.5-cc. portion of each reaction mixture was repeatedly applied on two pieces of filter paper (4×43 cm.), developed as before, and observed under ultraviolet rays. The crude enzyme solution from yellow-type mycelium was also treated in the same manner. As shown in Table V, the spots of 6-methyl-7-hydroxyribolumazine and riboflavin produced through 6,7-dimethylribolumazine were detected in the case of the enzyme solution from the yellow-type mycelium, but no spots of riboflavin and others derived from 4-ribitylamino-5-aminouracil were found in the enzyme solution from the leuco-type mycelium, as observed in its control test. The spot corresponding to riboflavin detected in the case of the enzyme solution from the yellow-type mycelium was cut out and extracted, and riboflavin in the extract was determined as before.

Table V. Chromatogram of the Products from 4-Ribitylamino-5-aminouracil and Acetoin as Substrate, and Amount of the Resulting Riboflavin

Type of strain	Rf					Riboflavin detected (γ)	Riboflavin formed (γ)	mole	(%)
Leuco-type	0.05 B	0.12 ab	0.16 BG			—		—	
Control	0.05 B	0.12 ab	0.16 BG			—		—	
Yellow-type	0.05 B	0.12 ab	0.16 BG	0.23 V	0.35 Y	22.2	7.6	2.02×10^{-8}	0.67
Control	0.05 B	0.12 ab	0.16 BG		0.35 Y	14.6			
		↑		↑	↑				
		4-Ribityl-amino-5-aminouracil		6-Methyl-7-hydroxy-ribolumazine	Riboflavin				

A similar experiment was conducted once more and this time quantitatively. A mixture of 0.1 cc. of the crude enzyme solution, 0.1 cc. of 4-ribitylamino-5-aminouracil solution ($2.05\times10^{-3}M$), and 0.1 cc. of acetoin ($2\times10^{-2}M$) was incubated at 37° for 3 hr. the reaction mixture was applied on two pieces of filter paper (4×43 cm.), and subjected to paper partition chromatography. The yellow-fluorescent spot at Rf 0.35 was cut out, extracted with 5 cc. of 2% saccharine solution, and riboflavin in the extract was determined by the lumiflavin method. As seen from the results in Table VI, there was found a clear difference between the enzyme solutions from the yellow-type and leuco-type mycelia, the latter producing no riboflavin.

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Table VI. Amount of Riboflavin produced from 4-Ribitylamino-5-aminouracil and Acetoin as Substrate

	Strain	Culture period (hr.)	Crude enzyme	Protein-N γ/0.3 cc. (N)	Riboflavin detected (γ)	Riboflavin formed μmole/0.3 cc. (L)		Specific activity L/N × 1000	
1)	Leuco-type	65	liquid	11.3	——	0		0	
			〃 control		——	0		0	
2)	〃	94	liquid	11.8	——	0		0	
			〃 control		——	0		0	
3)	〃	65	solution of dried powder, 30.4 mg./cc.	28.4	——	0		0	
			〃 control		——	0		0	
4)	〃	94	solution of dried powder, 26.8 mg./cc.	34.0	——	0		0	
			〃 control		——	0		0	
5)	Yellow-type	44	liquid	11.85	2.36 (R = 0.10) (mean value in 2 expt.)	0.66	0.00175	0.85	0.147
			〃 control		1.70 (R = 0.0) (mean value in 2 expt.)				
6)	〃	68	liquid	9.35	2.35 (R = 0.5) (mean value in 3 expt.)	0.98	0.0026	1.27	0.278
			〃 control		1.37 (R = 0) (mean value in 2 expt.)				
7)	〃	44	solution of dried powder, 25 mg./cc.	22.8	4.23 (R = 0.10) (mean value in 2 expt.)	0.75	0.00201	0.98	0.088
			〃 control		3.47 (R = 0.19) (mean value in 2 expt.)				
8)	〃	68	solution of dried powder, 25.4 mg./cc.	20.45	4.18 (R = 0) (mean value in 2 expt.)	1.24	0.00328	1.6	0.160
			〃 control		2.94 (R = 0.21) (mean value in 2 expt.)				

Summary

1) The leuco-type strain of *Er. ashbyii* was cultivated for 120 hours in the same manner as the yellow-type strain and about 24 g./L. (medium) of the wet mycelium was obtained. Use of corn-steep liquor instead of meat extract raised the yield, the quantity of the mycelium amounting to 40.5 g./L. in 94 hr.

2) The mycelium of the leuco-type strain and that of the yellow-type strain were extracted and the extracts were chromatographed to investigate the amino acids therein. It was found that the former contained various amino acids but not hydroxykynurenine, and in its instead it contained proline which was absent in the yellow-type strain.

3) Presence of riboflavin in the leuco-type strain was confirmed by paper partition chromatography and the amount of riboflavin in the spot was determined, finding ca. 3 γ/g. (wet mycelium).

4) A crude enzyme solution was prepared from the leuco-type and yellow-type strains, and the solutions were acted on 6,7-dimethylribolumazine. It was found that both enzyme systems have the ability to produce riboflavin and 6-methyl-7-hydroxyribolumazine, but the enzyme system from the yellow-type strain is a little more active than that from the leuco-type strain. The enzyme solutions were also acted on a mixture of 4-ribitylamino-5-aminouracil and acetoin. In this case, the solution from the yellow-type strain produced riboflavin and 6-methyl-7-hydroxyribolumazine, but the solution from the leuco-type strain yielded no such substances.

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