UDC 543.544 : 577.164.12

79. Mitsuko Asai, Toru Masuda, and Satoru Kuwada : Application of Chromatography. XLIII.*1 On the Mechanism of Enzymic Conversion of 6,7-Dimethylribolumazine to Riboflavin.

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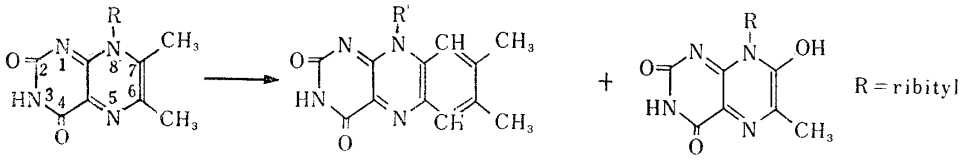
It was previously found[1] and reported*3 that reaction between 6,7-dimethylribolumazine and a crude enzyme solution prepared from the mycelium of *Eremothecium ashbyii* produced riboflavin and 6-methyl-7-hydroxyribolumazine as shown in Chart 1.



6,7-Dimethylribolumazine Riboflavin 6-Methyl-7-hydroxyribolumazine

Chart 1.

The method for preparing the crude enzyme solution was explained in detail and it was stated that the yield of riboflavin produced under a definite condition was 21.3% of 6,7-dimethylribolumazine used and that this yield was obtained without addition of any other substance as a carbon donor.[2]

Later, the enzyme solution was prepared according to the method reported previously and, after removing riboflavin in it by dialysis almost completely and adjusting its protein nitrogen to about 131.8 γ/cc., the enzyme solution was incubated with 6,7-dimethylribolumazine at 37° for one hour. A definite amount of the reaction mixture was subjected to paper partition chromatography (solvent : BuOH-EtOH-H_2O) and the resulting riboflavin spot at Rf 0.35 was determined by the lumiflavin method. The blue-fluorescent spot at Rf 0.22 and the green fluorescent spot at Rf 0.20 were respectively extracted with water and 6-methyl-7-hydroxyribolumazine produced was determined from the absorption at 345 mμ of the extract of the former spot and the unchanged 6,7-dimethylribolumazine from the absorption at 407 mμ of the latter spot. As a result, 23.4% of riboflavin, 41.2% of 6-methyl-7-hydroxyribolumazine, and 8.7% of 6,7-dimethylribolumazine were detected as shown in Table I in the experimental part.

On the other hand, similar experiments were conducted in the presence of diacetyl and pyruvate to observe their effect on the reaction and the results are shown in Table II. As seen from the results, addition of diacetyl considerably decreased the formation of riboflavin, while addition of pyruvate somewhat accelerated it. The results obtained by examination of the reaction mixtures by paper partition chromatography are shown in Table III. It will be noted from this table that when diacetyl was added, the amount of unchanged 6,7-dimethylribolumazine was remarkable and the formation of 6-methyl-7-hydroxyribolumazine was not observed, whereas when pyruvate was added, the amount of 6-methyl-7-hydroxyribolumazine was a little smaller and those of riboflavin and 6,7-dimethylribolumazine were about the same as when pyruvate was not added. In this chromatogram, the yellow spot at Rf 0.06 seemed to be of FAD originally contained

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*1 Part XLII : This Bulletin, **9**, 498 (1961).

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

*3 The result was reported at the meeting of the Vitamin Committee before publication in Ref. (1).

1) S. Kuwada, T. Masuda, T. Kishi, M. Asai : This Bulletin, **6**, 618 (1958).

2) S. Kuwada : Vitamins (Japan), **14**, 933 (1958).

in the crude enzyme solution, and the bluish green spot at Rf 0.10 closely resembled the dimeride of 4-ribitylamino-5-aminouracil (Rf 0.08; solvent: BuOH-EtOH-H_2O) described in Part XLI of this series[3] but it has not been fully identified yet. This spot was not detected when diacetyl was added.

The above results were not published until now because no conclusion was reached. Recently, Plaut[4] also attempted reaction of a pure enzyme prepared from *Ashbya gossypii* with 6,7-dimethylribolumazine *in vitro*, in the presence of about 20 kinds of carbon donors, but no substance accelerating the formation of riboflavin was found. It should especially be noted that when acetate [1,2-^{14}C] or uniformly labeled glucose was added, the resulting riboflavin had no radioactivity. Therefore, the methyl groups at 6- and 7-positions of 6,7-dimethylribolumazine were labeled with carbon-14 and a definite amount of the product was incubated with the above enzyme without addition of any carbon donor. Riboflavin and unchanged 6,7-dimethylribolumazine in the reaction mixture were separated by column chromatography and determined from their absorption or fluorescence. The molar specific radioactivity of the resulting riboflavin was also measured and furthermore, the distribution of radioactivity in the riboflavin was clarified from the formation of radioactive acetic acid by decomposition of riboflavin by the Kuhn-Roth method. From these results Plaut concluded that 6,7-dimethylribolumazine acts as a carbon donor as well as carbon acceptor in this reaction, as shown in Chart 2, and therefore one mole of riboflavin is produced from 2 moles of 6,7-dimethylribolumazine, the molar specific radioactivity of the resulting riboflavin becoming twice as strong as that of the original 6,7-dimethylribolumazine. If the reaction proceeds as Plaut presumed, the question would be in what form the residual pyrimidine part resulting by splitting off of four carbon atoms remain in the reaction mixture and this problem was said to be under investigation.

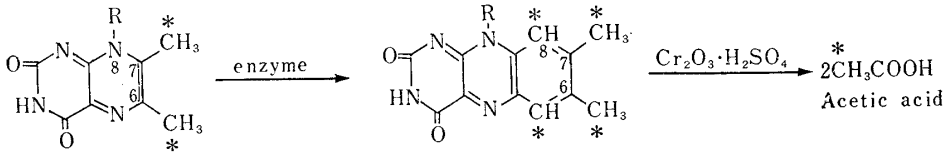


Chart 2.

When the report of Plaut is compared with the present authors' experience, it was doubtful whether he fully considered the meaning of the formation of 6-methyl-7-hydroxyribolumazine in this reaction. He submitted the reaction mixture to column chromatography on Magnesol to separate the unchanged 6,7-dimethylribolumazine, the resulting riboflavin, and 6-methyl-7-hydroxyribolumazine, and stated that the molecular ratio between the consumed 6,7-dimethylribolumazine and the resulting riboflavin was 2:1, but did not touch on the amount of 6-methyl-7-hydroxyribolumazine. In another report[5] he announced similar results and added that 3 moles of 6,7-dimethylribolumazine were consumed for formation of 1 mole of riboflavin, and that formation of 6-methyl-7-hydroxyribolumazine, besides riboflavin, was confirmed. The two reports, however, seem to contain an inconsistent or not fully deliberated point. Consulting the reports of Plaut, some experiments were carried out to elucidate some hitherto unclarified points, the results of which are described below.

3) S. Kuwada, T. Masuda, T. Kishi, M. Asai : *Ibid.*, 8, 798 (1960).
4) G.W.E. Plaut : J. Biol. Chem., 235, PC 41 (1960).
5) *Idem* : Federation Proc., **19**, 312 (1960).

First, the reaction between 6,7-dimethylribolumazine and the enzyme solution was effected in the presence and absence of diacetyl to give the results shown in Table IV (a), in which the method for preparing the enzyme solution, reaction conditions, separation of the resulting products by paper partition chromatography, and determination of them were all the same as described in the first part of this paper. As is evident from the table, when diacetyl was not added, total amount of the unchanged 6,7-dimethylribolumazine and the resulting riboflavin and 6-methyl-7-hydroxyribolumazine did not come up to 100% of the original substrate. In other words, a substance other than riboflavin and 6-methyl-7-hydroxyribolumazine must have been formed. In this case, however, if 2 moles of 6,7-dimethylribolumazine are consumed for formation of 1 mole of riboflavin as Plaut stated, the calculation may be made after doubling the percentage of riboflavin and the total amount reaches nearly 100%, as shown outside the table. Thus it became necessary to search for the substance, other than 6-methyl-7-hydroxyribolumazine, produced from the pyrimidine part which was formed by splitting off of four carbon atoms.

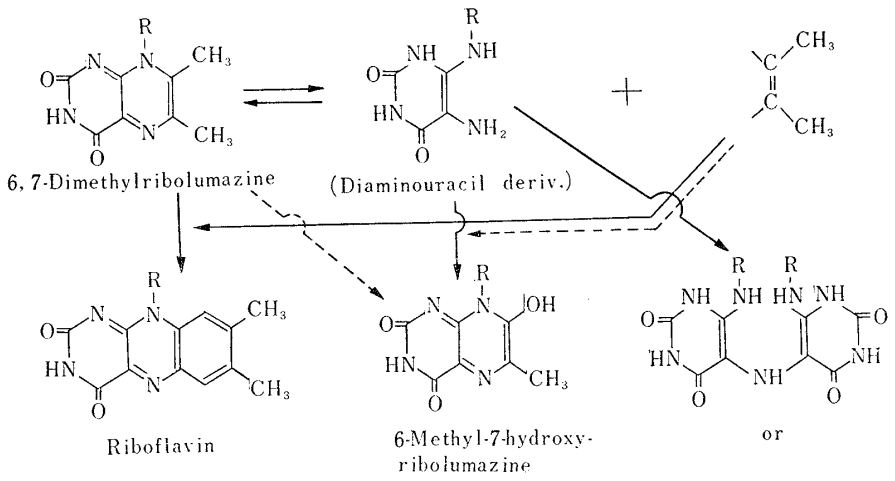
On the other hand, when diacetyl was added, the formation of riboflavin was smaller, as shown in Table II, the amount of the unchanged 6,7-dimethylribolumazine was large, and that of the resulting 6-methyl-7-hydroxyribolumazine was very small, as shown in Table IV (a), but the total amount of the unchanged 6,7-dimethylribolumazine and the resulting riboflavin and 6-methyl-7-hydroxyribolumazine amounted to about 100% of the original substrate. This may be explained by assuming that 2 moles of 6,7-dimethylribolumazine were consumed for formation of 1 mole of riboflavin and 1 mole of the unidentified substance, as mentioned before, but the latter reverted to 6,7-dimethylribolumazine by condensation with the added diacetyl, while its conversion to 6-methyl-7-hydroxyribolumazine was suppressed by diacetyl. The fact that the bluish green fluorescent spot at Rf 0.10, which was assumed to be of the dimeride of the pyrimidine compouud, was not detected in this case may also be explained by assuming that its formation was inhibited by the above-mentioned condensation with diacetyl. The experiments in Table IV (b) were carried out using another lot of the enzyme solution to confirm the results of (a) and to observe the effect of acetoin and pyruvate on the reaction. Whether acetoin was added or not, the results were almost the same, and therefore it seems to exert no effect on the enzymic reaction. When pyruvate was added, the amount of unchanged 6,7-dimethylribolumazine was markedly great, if not so large as when diacetyl was added, and the amount of the resulting riboflavin was about the same as when pyruvate was not added. This is the result of only one of many similar experiments, but the others also showed about the same tendency, and therefore it is premature to give a conclusion as to the effect of the added pyruvate.

As is obvious from the foregoing, there still remain some points to be clarified, but the mechanism shown in Chart 3 may be considered for this enzymic reaction.

In the above consideration, the mechanism for formation of 6-methyl-7-hydroxyribolumazine still remains unsettled. However, two routes are considered from the quantitative relation of the products, as shown in Chart 3, in one of which the methyl group at 7-position of 6,7-dimethylribolumazine may be changed to hydroxyl group, and in the other the C_4 substance split off from 6,7-dimethylribolumazine may be converted to a C_3 substance, which then condenses with the residual pyrimidine part to produce 6-methyl-7-hydroxyribolumazine. From the fact that addition of diacetyl suppressed the formation of 6-methyl-7-hydroxyribolumazine, the latter route seems to be more probable.

The experiments reported in this paper were all conducted *in vitro*, using cell-free enzyme solutions. Since, however, the mycelium of *Er. ashbyii* may contain many unidentified substance besides a large quantity of acetoin and pyruvate,[6] it is unthinkable

6) T. Masuda : This Bulletin, 5, 136 (1957).



Quantitative relation of the resulting products

6,7-Dimethylribolumazine (2 moles) ⟶ Riboflavin (1 mole) + Diaminouracil deriv. (1 mole)

6,7-Dimethylribolumazine (1 mole) ⟶ 6-Methyl-7-hydroxyribolumazine(1 mole)

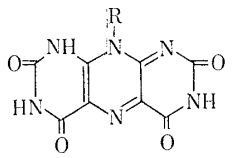


Chart 3.

that the same enzymic conversion as mentioned before takes place in the living body. It has so far been assumed[6] that the reverse reaction of the degradation of 6,7-dimethylribolumazine into the pyrimidine part and C_4 takes place as the final step in the biosynthesis of 6,7-dimethylribolumazine in living cells, and therefore, if this assumption is right, there must be a substance giving C_4 for the formation of 6,7-dimethylribolumazine. It would be interesting to investigate under what conditions and in what way the reversible enzymic conversion is effected in the living body.

Experimental

Preparation of Enzyme Solution——The enzyme solution was prepared from the mycelium of *Er. ashbyii* according to the method described in Part XXXVI[1] of this series. In the present work a lot containing 131.8 γ/cc. of protein-N was used in most cases.

Conversion 6,7-Dimethylribolumazine to Riboflavin——A mixture of 0.5 cc. of an aqueous solution of 6,7-dimethylribolumazine ($3.1\times10^{-3}M$) and 0.5 cc. of the enzyme solution was incubated at 37° for 1 hr. and a definite amount of the reaction mixture was subjected to paper partition chromatography by the method reported previously.[1] The green-fluorescent spot at Rf 0.20 (a), the purple-fluorescent spot at Rf 0.22 (b), and the yellow-fluorescent spot at Rf 0.35 (c) were each extracted with a definite amount of water, and 6,7-dimethylribolumazine in the extract (a), 6-methyl-7-hydroxyribolumazine in the extract (b), and riboflavin in the extract (c) were determined from absorption at 407 mμ and 345 mμ, and by the lumiflavin method, respectively. The control was the same enzyme solution but its enzymic activity was destroyed in advance by heating at 100° for 10 min. The amounts of the unchanged 6,7-dimethylribolumazine and the resulting riboflavin and 6-methyl-7-hydroxyribolumazine

Table I. Action of Crude Enzyme of *Er. ashbyii* on 6,7-Dimethylribolumazine

	Control (mole)	With enzyme soln. incubated at 37° 1 hr.	Percentage to substrate
6,7-D (unchanged)	1.505×10^{-6}	1.32×10^{-7}	8.76
FR (produced)	0	3.52×10^{-7}	23.4
6,7-MH (unchanged)	0	6.2×10^{-7}	41.2

6,7-D=6,7-Dimethylribolumazine
FR=Riboflavin
6,7-MH=6-Methyl-7-hydroxyribolumazine

are shown in Table I and the figures in the last column of the table indicate their percentage to the original substrate.

Comparison of Experiments in the Presence and Absence of Carbon Component——With emphasis on the production of riboflavin, a mixture of the substrate and the enzyme solution, and the mixture plus diacetyl or pyruvate were treated as above and the results are shown in Table II.

Table II. Formation of Riboflavin under Various Conditions

	Amount of riboflavin formed		
	γ	Mole ($\times 10^{-9}$)	Percentage to substrate
Enzyme soln. + 6,7-D (1.25×10^{-7} mole)	7.65	20.4	16.3
〃 + 〃	7.25	19.3	15.4
〃 + 6,7-D + diacetyl (1.96×10^{-6} mole)	5.20	13.8	11.1
〃 + 〃 + 〃	4.90	13.0	10.4
〃 + 〃 + pyruvate (1.98×10^{-6} mole)	8.75	23.2	18.5
〃 + 〃 + 〃	8.35	22.2	17.8

Each reaction mixture was subjected to paper partition chromatography and the resulting chromatograms were identified, giving the results shown in Table III. Description of each spot is given in the main text.

Table III. Reaction between 6,7-Dimethylribolumazine and Enzyme Solution (Examination by paper partition chromatography)

	Rf (Solvent : BuOH-EtOH-H_2O)					
1) Heat treated enzyme soln. + 6,7-D	0.35 Y ±		0.20 G ##		0.06 Y ±	0.00 f ±
2) Enzyme soln. + 6,7-D	0.33 Y ##	0.22 V ##	0.20 G +	0.10 GB ±	0.07 Y ±	0.00 f ±
3) 〃 + 〃 + Diacetyl	0.33 Y ##		0.19 G ##		0.08 Y ±	0.00 f ±
4) 〃 + 〃 + Pyruvate	0.33 Y ##	0.22 V +	0.20 G +	0.10(?) GB ±	0.06 Y ±	0.00 f ±
	↑ FR	↑ 6,7-MH	↑ 6,7-D		↑ FAD	

f; fluorescence, Y; yellow fluorescence, G; green fluorescence, GB; green-blue fluorescence, V; violet fluorescence, ±～##; intensity of fluorescence.

Reëxamination of the Enzymic Reaction——Many experiments were conducted to reëxamine the enzymic reaction stated before, but it is difficult to describe all of them because the concentrations of the substrate and the enzyme solution and other conditions were not necessarily the same. In the experiments of Table IV (a and b), a mixture of 1 cc. each of the test solutions was incubated at 37° for 1～3 hr., the reaction mixture was chromatographed, and each of the separated spots was extracted and submitted to determination. Table IV (a) shows the comparison of results in two experiments, in one of which a mixture consisting of only the substrate and the enzyme solution was used, and in the other the mixture plus diacetyl was employed. Table IV (b) indicated the results of experiments in which another lot of the enzyme solution was used, and the same mixtures as in (a) and the mixture plus acetoin or pyruvate was employed as the reactants.

Summary

It was previously found[1] that reaction between 6,7-dimethylribolumazine and a crude enzyme prepared from the mycelium of *Er. ashbyii* produced riboflavin and 6-methyl-7-hydroxyribolumazine and reported[2] that the enzymic reaction yielded a considerable amount of riboflavin without addition of any carbon donor. Later, the products in the enzymic reaction were investigated by paper partition chromatography and a bluish green fluorescent spot with Rf 0.10 was detected besides the known substances, and the newly found substance was assumed to be the dimeride of 4-ribitylamino-5-aminouracil described in part XLI of this series.

TABLE IV(a). Progress of the Enzymic Reaction *in vitro* under Various Conditions

	Enzyme soln. (106 γ/cc.)* + 6,7-D (3.0×10^{-6} mole)						Enzyme soln. (106 γ/cc.) + 6,7-D (3.0×10^{-6} mole) + diacetyl (1.96×10^{-5} mole)					
	1 hr.		2 hr.		3 hr.		1 hr.		2 hr.		3 hr.	
	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%
6,7-D	2.18	72.7	1.78	59.3	1.47	49	2.75	91.7	2.84	94.6	2.75	91.7
FR	0.282	9.4 (18.8)	0.410	13.65 (27.30)	0.427	14.23 (28.46)	0.129	4.3	0.136	4.53	0.19	6.33
6,7-MH	0.298	9.93	0.458	15.75	0.61	20.3	0.068	2.26	0.016	0.53	0	0
Total	2.76	92.03 (101.43)	2.648	88.7 (101.35)	2.507	83.58 (97.76)	2.947	98.26	2.992	99.96	2.94	98.03

* Content of protein nitrogen

TABLE IV(b). Progress of the Enzymic Reaction *in vitro* under Various Conditions

	Enzyme soln. (216 γ/cc.) + 6,7-D (2.88×10^{-6} mole)						Enzyme soln. (216 γ/cc.) + 6,7-D (2.88×10^{-6} mole) + diacetyl (2.0×10^{-5} mole)					
	1 hr.		2 hr.		3 hr.		1 hr.		2 hr.		3 hr.	
	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%
6,7-D	1.36	47.2	1.045	36.3	0.199	6.14	2.6	90.2	2.67	92.7	2.47	85.8
FR	0.5	17.36 (34.72)	0.553	19.2 (38.4)	0.595	20.65 (41.30)	0.235	8.15	0.261	9.06	0.276	9.55
6,7-MH	0.53	18.4	0.74	25.7	1.545	53.4	0	0	0	0	0.064	2.2
Total		82.96 (100.32)		81.2 (100.4)		80.15 (100.34)		98.35		101.76		97.55

	Enzyme soln. (216 γ/cc.) + 6,7-D (2.88×10^{-6} mole) + acetoin (5.06×10^{-5} mole)						Enzyme soln. (216 γ/cc.) + 6,7-D (2.88×10^{-6} mole) + pyruvate (2.88×10^{-5} mole)					
	1 hr.		2 hr.		3 hr.		1 hr.		2 hr.		3 hr.	
	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%	10^{-6} mole	%
6,7-D	1.397	48.5	1.24	43.0	0.541	18.78	2.12	73.6	2.15	74.6	2.06	71.5
FR	0.524	18.3 (36.6)	0.618	21.45 (42.9)	0.686	23.8 (47.6)	0.469	16.3 (32.6)	0.506	17.55 (35.1)	0.526	18.25 (36.5)
6,7-MH	0.44	15.3	0.559	19.4	1.115	38.7	0.118	4.1	0.197	6.8	0.15	5.2
Total		82.1 (100.4)		83.85 (105.3)		81.28 (105.08)		94.0 (110.3)		98.95 (116.5)		94.95 (113.2)

Recently, Plaut[4] discussed the mechanism of the formation of riboflavin by reaction between 6,7-dimethylribolumazine and an enzyme prepared from *Ashbya gossypii*. With a view to confirming the theory of Plaut, the above-mentioned reactions were repeated, and unchanged 6,7-dimethylribolumazine, the resulting riboflavin, and 6-methyl-7-hydroxyribolumazine were separated by paper partition chromatography and determined. The results seemed to be well explained when it is considered, together with the formation of a large amount of 6-methyl-7-hydroxyribolumazine, that one mole of riboflavin is produced from two moles of 6,7-dimethylribolumazine as pointed out by Plaut. Although the identity of the fluorescent spot with Rf 0.10 and the mechanism for formation of 6-methyl-7-hydroxyribolumazine are not yet fully clarified, some findings were obtained on the effect of the addition of carbon components such as diacetyl, acetoin, and pyruvate on the enzymic reaction.

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