the position is vacant) to the ether linkage, as in anisole,[5] phenethole,[6] and diphenyl ether.[5,7] In substituted alkyl phenyl ethers, the ether linkage is split to give a phenol, i.e. process (b), as is known by the examples of *p*-iodo-,[8] *p*-chloro-,[9] and *p*-nitro-anisoles,[9] 4-methoxy-[10] and 4, 4'-dimethoxyphenyl ethers,[11] phenetidine,[12] and phenacetin.[13]

A combination of (a) and (b) would occur in the production of dihydroxyphenol as in process (c).

According to the present conditions, it appears that the process (b) will be the case in the metabolism of herniarin. This opinion was confirmed by the fact that umbelliferone was produced *in vivo* as a result of demethylation of the herniarin administered.

From the determination of the metabolites, it was known that herniarin was converted into umbelliferone to the extent of 29.94% of the amount administered. Umbelliferone was excreted mainly in the free state (20.35%), though a small portion (9.59%) was combined. Conjugated umbelliferone was composed of 8.64% of glucuronide and 1.08% of ethereal sulfate.

It may be noted that evidences for the cleavage of lactone ring and biological hydroxylation have not been found.

From a quantitative point of view, the recovery of herniarin in the urinary metabolites was no more than 30% of the dose (200 mg./kg.). No information is available at present as to the remainder of metabolites.

Summary

The fate of herniarin in the rabbit was studied. The chief metabolite is umbelliferone but a small amount of unchanged herniarin was excreted, and both metabolites were estimated quantitatively. No evidences for biological hydroxylation or lactone-ring cleavage was obtained.

(Received May 12, 1958)

5) H. G. Bray, S. P. James, W. V. Thorpe, M. R. Wasdell: Biochem. J., **54**, 547(1953).
6) A. Kossel: Z. physiol. Chem., **4**, 296(1880); *ibid.*, **7**, 292(1883); V. Lehmann: *Ibid.*, **13**, 181(1889).
7) S. W. Stroud: J. Endocrinol. (London), **2**, 55(1940)
8) F. Röhmann: Biochem. Zentr., **3**, 688(1905).
9) H. G. Bray, V. M. Caddock, W. V. Thorpe: Biochem. J., **60**, 225(1955).
10) S. W. Stroud: Nature, **144**, 245(1939).
11) S. W. Stroud: *Ibid.*, **146**, 166(1940).
12) T. N. Smith, R. T. Williams: Biochem. J., **44**, 250(1949).
13) T. N. Smith, R. T. Williams: *Ibid.*, **44**, 239(1949).

UDC 547.854.4'861.6 : 582.284 : 545.84

104. Toru Masuda, Toyokazu Kishi, Mitsuko Asai, and Satoru Kuwada: Application of Chromatography. XXXV.* On the Biochemical Significance of 6-Methyl-7-hydroxyribolumazine in the Culture of *Eremothecium ashbyii*.

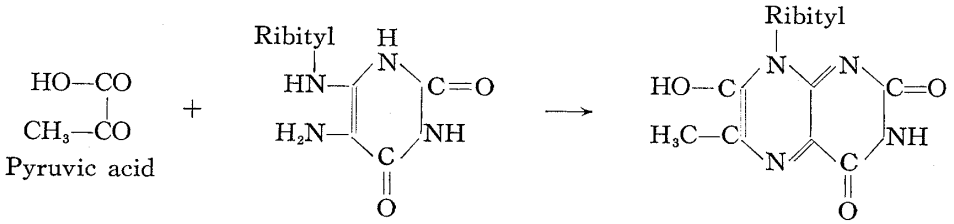
(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.***)

One of the authors (Masuda) formerly isolated a green and a violet fluorescent substances from the mycelium and broth of *Eremothecium ashbyii*[1] and they were respectively named 6, 7-dimethylribolumazine and 6-methyl-7-hydroxyribolumazine.[2] As riboflavin was pro-

* Part XXXIV: This Bulletin, **6**, 291(1958).
** Juso-nishino-cho, Higashiyodogawa-ku, Osaka (増田 亨, 貴志豊和, 浅井満子, 桑田 智).
1) T. Masuda: This Bulletin, **3**, 434(1955).
2) S. Kuwada, T. Masuda, T. Kishi, M. Asai: *Ibid.*, **6**, 447(1958).

duced from 6,7-dimethylribolumazine *in vitro* and *in vivo* as well, the latter compound was considered as an intermediate in the biosynthesis of riboflavin.[3,4]

This compound seems to be formed from a purine compound through 4-ribitylamino-5-aminouracil,[3] and it can also easily be assumed that this uracil compound would react with pyruvic acid to give 6-methyl-7-hydroxyribolumazine.



Although it is not yet confirmed experimentally, the above assumption appears certain from the fact that pyruvic acid is present in the mycelium and broth of *Er. ashbyii*, that addition of pyruvic acid during the cultivation of *Er. ashbyii* tends to decrease the formation of riboflavin but to increase the formation of 6-methyl-7-hydroxyribolumazine, and that in the normal cultivation of *Er. ashbyii*, 6-methyl-7-hydroxyribolumazine increases in parallel with increase of riboflavin, while 6,7-dimethylribolumazine is produced in a very small quantity, showing that 6-methyl-7-hydroxyribolumazine is a final product in the metabolism.

Experimental

1) **Quantitative Determination of 6,7-Dimethylribolumazine in the Mycelium of** *Er. ashbyii*—It was conducted according to the report of Masuda.[4]

2) **Quantitative Determination of 6-Methyl-7-hydroxyribolumazine in the Mycelium of** *Er. ashbyii*—An amount of 1~8 g. of the wet mycelium was extracted with 20~50 cc. of water at 80° and the extract was adjusted to a definite volume. A portion of 0.05 cc. of the extract was applied on the middle of a paper strip, 4×45 cm., and migrated in the Theorell buffer (pH 6.0, μ=0.1) at 300 V for 4 hrs. The violet fluorescent spot on the side of anode, detected under ultraviolet light, was cut out and extracted with 5 cc. of water. The intensity of fluorescence of the extract, measured by the Kotaki fluorometer, was compared with that of the control. The control was prepared by treating 0.05 cc. of a 104 γ/cc. solution of crystalline 6-methyl-7-hydroxyribolumazine as above.

3) **Pyruvic Acid in the Mycelium and Broth of** *Er. ashbyii*—*Er. ashbyii* was cultivated for 4 days with shaking, 45 g. of the wet mycelium separated by filtration was extracted with water at ca. 80°, and the extract was concentrated to about 10 cc. *in vacuo*. A volume of 100 cc. of the filtered broth was also concentrated to about 5 cc. under a reduced pressure. Each of the concentrates, after being applied on Whatman No. 1 filter paper (1.5×45 cm.), was subjected to electrophoresis at 300 V in a Theorell buffer (pH 7.0, μ=0.1), and the dried pherograms were sprayed with 5% sodium nitroprusside solution and 30% NaOH solution to produce red-brown spots at the place shown in Table I.

TABLE I.

Substance	Migration distance (cm.)				
Mycelium	−1	−2 (+)	+10 (±)		
Culture Filtrate	±0	−1.8 (+)	+10 (+)		(+), (±) Intensity of color
Pyruvic acid			+10		
Acetoin	−1.5				

However, the spot produced by the extract of the mycelium at +10 cm. was faint in color.

About 1.5 L. of the above filtered broth was concentrated to 100 cc., a solution of 0.1 g. of 2,4-dinitrophenylhydrazine in 50 cc. of 2*N* HCl was added, and the mixture, after being heated at 50° for 30 mins., was allowed to cool by standing for 1 hr. The reaction mixture was extracted by shaking several times with AcOEt in a separatory funnel and the combined extract was concentrated under a reduced pressure. The concentrate was developed on a filter paper with EtOH-BuOH-0.5*N* NH_4OH (10 : 70 : 20) to give yellow spots at distances shown in Table II. From this result it seems certain that pyruvic acid is present in the broth.

3) T. Masuda, T. Kishi, M. Asai: *Ibid.*, **6**, 291 (1958).
4) T. Masuda: *Ibid.*, **5**, 136 (1957).

TABLE II.

Substance	Rf value				
Reaction mixture of broth	0.06	0.22	0.45	0.62	0.77
Pyruvic acid 2,4-Dinitrophenylhydrazone			0.45		
α-Ketoglutaric acid 2,4-Dinitrophenylhydrazone[5]	0.08～0.15				
Oxalacetic acid 2,4-Dinitrophenylhydrazone[5]		0.19～0.29			
Acetoacetic acid 2,4-Dinitrophenylhydrazone[5]			0.46～0.64		

4) Effect of Pyruvic Acid on the Cultivation of *Er. ashbyii*—Eight culture flasks containing 100 cc. of the basic medium[6] in each were used as the control group, and eight culture flasks containing the same volume of the same medium and 1.0 g. of pyruvic acid as the pyruvic acid-added group. Each group was adjusted to pH 7.0, sterilized, and, after being inoculated with 48-hr.-old seed culture of *Er. ashbyii*, subjected to shaking culture for 66 hrs. Riboflavin and 6-methyl-7-hydroxyribolumazine in the mycelium and broth of both groups were determined and gave the results shown in Table III.

TABLE III.

Standard group

Sample No.	Wt. of mycelium (g./L.)	Amt. of riboflavin in mycelium (mg./L.)	Amt. of riboflavin in culture filtrate (mg./L.)	Total riboflavin (mg./L.)	Amt. of 6-methyl-7-hydroxyribolumazine(mg./L.)
1	19.2	76.5	60.6	137.1	13.9
2	21.1	63.5	61.2	124.7	13.8
3	23.3	63.4	59.6	123.0	12.1
4	24.4	64.0	63.2	127.0	12.6
5	21.1	66.5	76.8	143.3	12.4
6	22.2	62.5	62.8	125.3	11.5
7	24.7	69.0	59.2	128.3	13.1
8	20.4	62.5	70.8	133.3	10.6
			average·····	130.3	12.5

Pyruvic acid-added group

Sample No.	Wt. of mycelium (g./L.)	Amt. of riboflavin in mycelium (mg./L.)	Amt. of riboflavin in culture filtrate (mg./L.)	Total riboflavin (mg./L.)	Amt. of 6-methyl-7-hydroxyribolumazine(mg./L.)
9	15.9	45.2	45.2	90.4	19.4
10	15.5	38.3	46.8	85.1	18.6
11	16.6	48.6	46.0	94.6	17.5
12	16.6	42.8	43.2	86.0	17.5
13	18.9	55.5	45.6	101.1	19.9
14	17.0	47.4	43.6	91.0	17.2
15	18.2	51.0	44.4	95.4	19.3
16	16.5	57.5	45.2	102.7	15.3
			average·····	93.3	18.1

5) Variation of Riboflavin, 6,7-Dimethylribolumazine, and 6-Methyl-7-hydroxyribolumazine in the Culture of *Er. ashbyii*—In 30 L. of the basic medium described in 4), 72-hr.-old seed culture of *Er. ashbyii* was cultivated. During the cultivation, a definite amount of the broth was taken out at regular intervals and 1～10 g. of the mycelium separated from it was extracted with water at 80°. 6-Methyl-7-hydroxyribolumazine in the extract was determined by the method mentioned before and the riboflavin and 6,7-dimethylribolumazine by Masuda's method.[4] The results are shown only as a graph (Fig. 1) indicating the relationship between the amount of the substance and culture time. The same determination on the filtered broth was unsuccessful because of a small amount of the substances.

5) M. F. S. ElHawary, R. H. S. Thompson: Biochem. J., **53**, 340(1953).
6) Polypeptone 0.8%, bonito extract 0.8%, $MgSO_4 \cdot 7H_2O$ 0.1%, KH_2PO_4 0.2%, NaCl 0.1%, and glucose 2.0%.

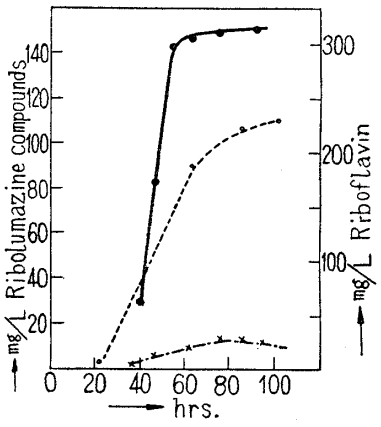


Fig. 1.
—•—•— Riboflavin
---•---•--- 6-Methyl-7-hydroxyribolumazine
--×--×-- 6, 7-Dimethylribolumazine

Discussion

The authors previously assumed the route for the biosynthesis of riboflavin in the culture of *Er. ashbyii* as purine compound (I) ⟶ uracil compound having a ribityl group (II) $\xrightarrow{\text{acetoin or diacetyl}}$ 6, 7-dimethylribolumazine (III) $\xrightarrow{\text{acetoin}}$ riboflavin (IV). Efforts have been made to confirm this assumption experimentally.

Of the compounds found in the route those which have so far been actually detected in *Er. ashbyii* are (I), (III), and (IV), (II) being an imaginary substance as yet. As (I) there have been detected adenosine, ATP, and the like, but it is still unknown how these compounds are converted to (II).

The mycelium as well as the broth of *Er. ashbyii* contains pyruvic acid, and the general concept as to relation between compounds of this series in yeast, microörganisms, and animal tissues is shown in Chart 1.

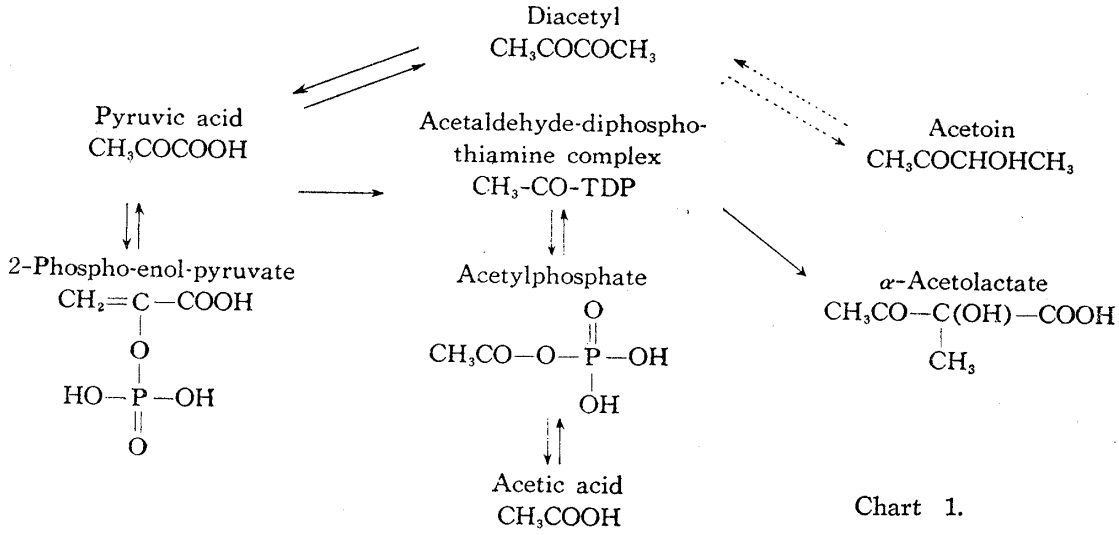


Chart 1.

Considering that *Er. ashbyii* actually contains pyruvic acid and acetoin, it seems natural that oxidation and reduction are effected between them.

On the other hand, diaminouracil condenses with pyruvic acid in an acidic medium to produce 6-hydroxy-7-methyllumazine, but addition of hydrazine in this case yields the 6-methyl homolog.[3] Though reaction of 2,4,5-triamino-6-hydroxypyrimidine with methylglyoxal gives 2-amino-4-hydroxy-7-methylpteridine, the same reaction in the presence of hydrazine affords the 6-methyl isomer.[7] It is dangerous to think that such reactions as above which were

7) H. S. Forrest, J. Walker: J. Chem. Soc., **1949**, 2077.

conducted *in vitro* may be effected in the body of microörganisms, but as blocking the carbonyl of pyruvic acid with hydrazine produced the 6-methyl homolog, formation of 6-methyl-7-hydroxyribolumazine *in vivo*, probably through the process mentioned in the beginning, is understandable because pyruvic acid in living cells always exists in the form of enolic phosphate and the activity of its carbonyl group is thereby blocked.

Thus considered, as 4-ribitylamino-5-aminouracil reacts with acetoin or diacetyl to produce 6,7-dimethylribolumazine and with pyruvic acid to give 6-methyl-7-hydroxyribolumazine, it is admissible that in the culture of *Er. ashbyii*, addition of pyruvic acid decreases the formation of riboflavin because the route from 4-ribitylamino-5-aminouracil to riboflavin is thereby blocked, but increases the formation of 6-methyl-7-hydroxyribolumazine.

Amounts of riboflavin and the two ribolumazine derivatives produced in the normal culture of *Er. ashbyii* were compared and, as a result it was found that 6,7-dimethylribolumazine does not increase in paralell with riboflavin probably because it is an intermediate of the latter, but the amount of 6-methyl-7-hydroxyribolumazine increased as it is a final product in this metabolism.

Studies are still under way on the relation between the two ribolumazine derivatives. The authors gratefully acknowledge the technical assistance of Mr. Yutaka Shiraishi in the cultivation of *Er. ashbyii*.

Summary

When *Er. ashbyii* was cultivated, pyruvic acid (V) was detected in the mycelium as well as in the broth. Addition of (V) in the cultivation medium, however, checked the formation of riboflavin (IV) and increased the production of 6-methyl-7-hydroxyribolumazine (VI). Formation of (VI) seems to be due to the reaction of (V) with 4-ribitylamino-5-aminouracil which is assumed to be an intermediate of (IV).

Er. ashbyii was cultivated in the basic medium, and determination was conducted on the (IV), (VI), and 6,7-dimethylribolumazine (III) in the samples collected at regular intervals. As a result, it was found that (III) was produced in a very small quantity, compared with (IV), and (VI) in a rather large quantity. This result is well explained by assuming that (IV) is formed through (III), and (VI) is a final product in this metabolism.

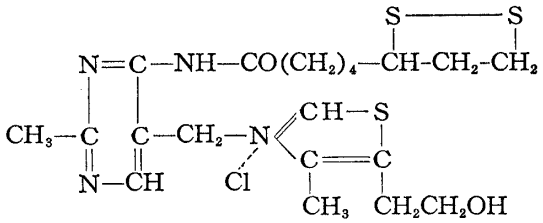
(Received May 15, 1958)

UDC 577.164.11

105. Shigeru Yoshida and Rinji Takasaki: Studies on the Allied Compounds of Vitamin B_1. XXII.[1] Acylation of Thiothiamine and Its Derivatives.[2]

(*Takamine Research Laboratory, Sankyo Co., Ltd.**)

Since the discovery by Reed and others[3] of lipothiamide (I), in which *a*-lipoic acid is bonded to the amino in 4-position of the pyrimidine ring in thiamine, the substance has been considered as taking important part in the decarboxylation of pyruvic acid *in vivo*.



(I)

* Nishi-Shinagawa, Shinagawa-ku, Tokyo (吉田茂, 高崎林治).

1) Part XXI: This Bulletin, **5**, 320(1957).
2) Paper presented at the 9th Annual Convention of the Pharmaceutical Society of Japan, Fukuoka, 1956.
3) L. J. Reed, B. G. DeBusk: J. Biol. Chem., **199**, 811, 873(1952); *idem.*: J. Am. Chem. Soc., **74**, 3964, 4727(1952).