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]

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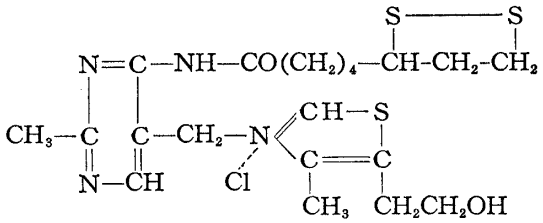
Since the discovery by Reed and others[3] of lipothiamide (I), in which *a*-lipoic acid is bonded to the amino in 4-position of the pyrimidine ring in thiamine, the substance has been considered as taking important part in the decarboxylation of pyruvic acid *in vivo*.



(I)

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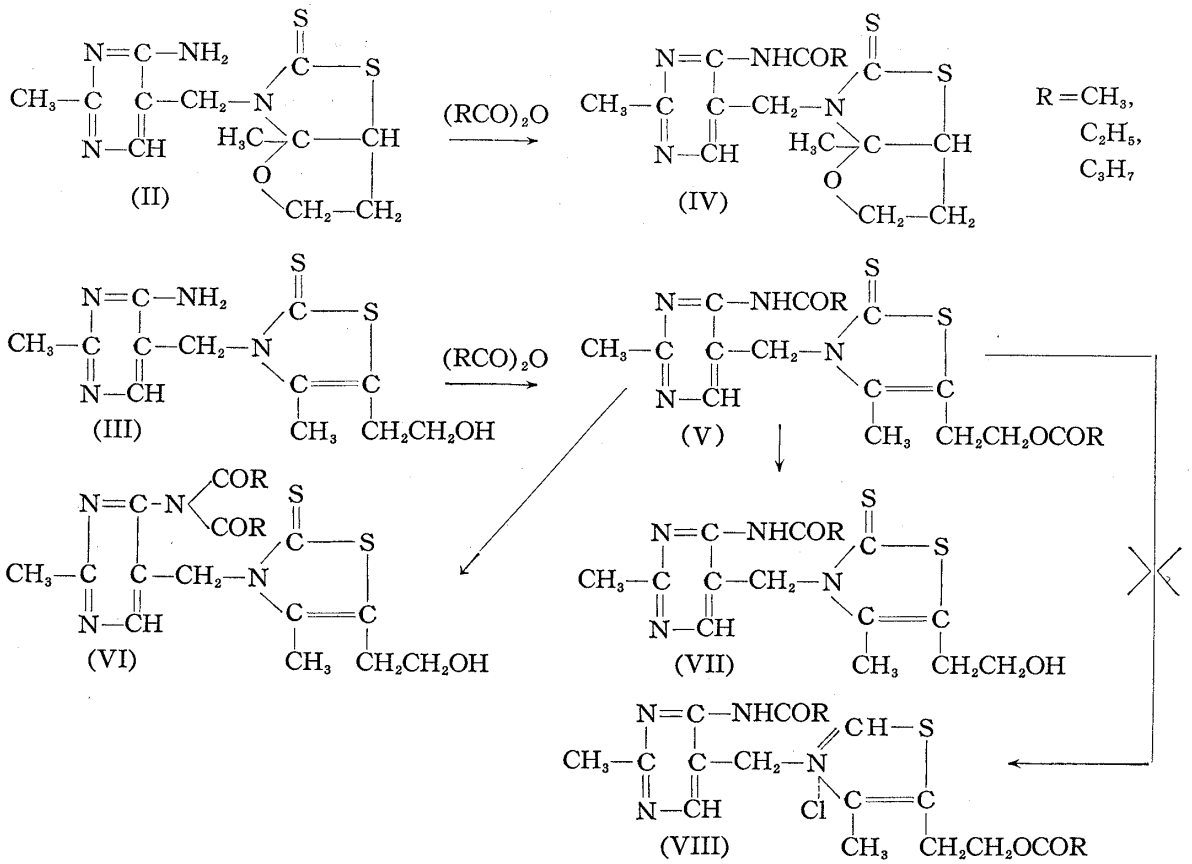
1) Part XXI: This Bulletin, **5**, 320(1957).
2) Paper presented at the 9th Annual Convention of the Pharmaceutical Society of Japan, Fukuoka, 1956.
3) L. J. Reed, B. G. DeBusk: J. Biol. Chem., **199**, 811, 873(1952); *idem.*: J. Am. Chem. Soc., **74**, 3964, 4727(1952).

Although the N-acylated thiamines are thought to be biologically important, neither lipothiamide nor N-acylated thiamines have yet been synthesized.

Consequently, as a preliminary for the syntheses of N-acylated thiamines, acylation of the stable 3-(2-methyl-4-amino-5-pyrimidylmethyl)-3a-methyl-perhydrofuro [2, 3-*d*] thiazole-2-thione (II) (hereinafter abbreviated as perhydrofurothiothiamine) and of thiothiamine (III) was attempted.

On heating (II) and (III) with acid anhydride at 70~100° for 2~3 hours, they respectively formed compounds (IV and V) acylated in the amino group at 4-position in (II) and (III), in almost quantitative yield. In the case of (III), esterification also occurred at the same time to produce N,O-diacetylated compound.



Ultraviolet absorption spectra of the starting materials and their acylated products are given in Figs. 1 and 2, and in Tables I and II. As is indicated in Fig. 1 and Table I, acylation of perhydrofurothiothiamine results in the shift of absorption at 237 mμ due to pyrimidine ring toward longer wave length by about 2 mμ, while the absorption at 278 mμ due to the perhydrofurothiazole ring[4] shifts toward a shorter wave-length region by 5 mμ. In acylated thiothiamines (V), as indicated in Fig. 2 and Table II, the absorption at 232 mμ shifts to 261 mμ but that at 324 mμ[4] shows no change.

TABLE I. Ultraviolet Absorption Maxima (in EtOH)

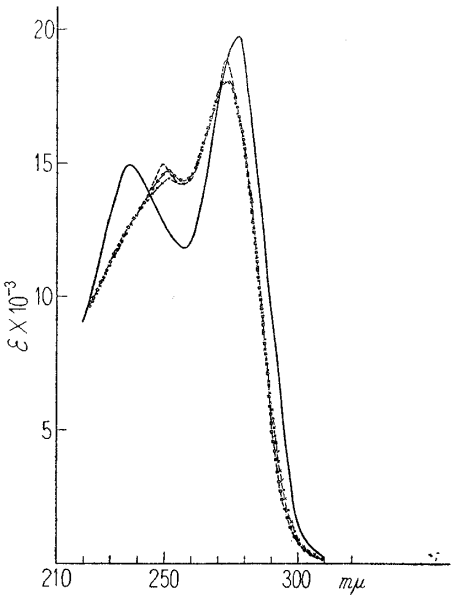
Compd. No.		mμ
(II)		237,278
(IV)	R=CH_3	249,273
	R=C_2H_5	251,273
	R=C_3H_7	253,272

TABLE II. Ultraviolet Absorption Maxima (in EtOH)

Compd. No.		mμ
(III)		232,324
(V)	R=CH_3	261,324
	R=C_2H_5	261,324
	R=C_3H_7	261,324
(VI)	R=CH_3	261,324

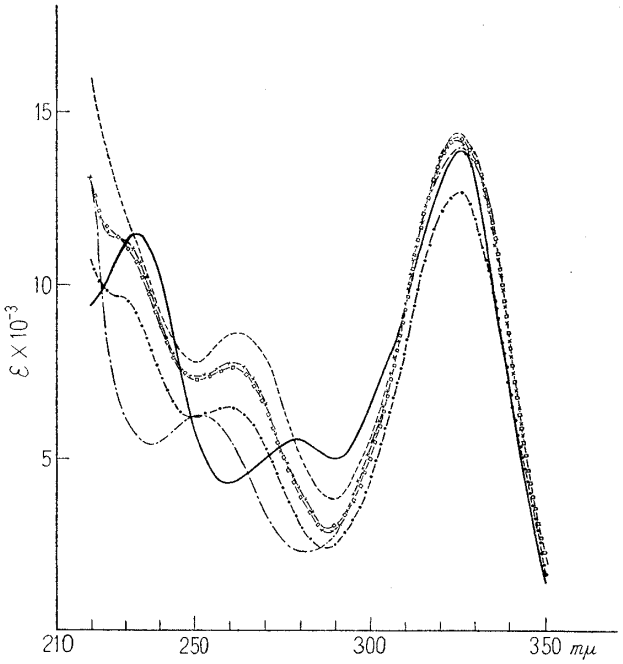
4) S. Yoshida, W. Ishizuka: Yakugaku Zasshi, **74**, 335(1954).

Fig. 1. Ultraviolet Absorption Spectra (in EtOH)



—— (II)
---- (IV) R=CH_3
-o-o- (IV) R=C_2H_5
-×-×- (IV) R=C_3H_7

Fig. 2. Ultraviolet Absorption Spectra (in EtOH)



—— (III)
-o-o- (V) R=CH_3
-□-□- Pseudo compound
---- (V) R=C_2H_5
-×-×-×- (V) R=C_3H_7
—·—·— (VI)

Infrared absorption spectra are listed in Table III. The compounds (IV) and (V) exhibit the N-H stretching band at around 3320 cm^{-1} and a primary amide I band at 1700~1720 cm^{-1}.

Table III. Infrared Spectra

Compd. No.		N-H Stretching vibration (cm^{-1})	C=O Stretching vibration (cm^{-1})	
			Ester band	Amide band
(IV)	R=CH_3	3320		1706
	R=C_2H_5	3320		1712
(V)	R=CH_3	3125	1740	1718
(VI)	R=CH_3		1733	1718, 1706
(VII)	R=C_2H_5	3290		1706

On heating (V : R=CH_3) with acetic anhydride in the presence of pyridine, white crystals (VI), m. p. 113°, were obtained and this substance exhibited two amide bands at 1718 and 1706 cm^{-1} [5] identical with the N, N-diacetyl compound (VI) obtained earlier by Uyeo and others.[6]

The acyl groups in the N-acylated compounds (IV and V) are extremely labile to acids and are liberated even on warming these compounds with 5% hydrochloric acid for about 30 minutes. In the case of (IV : R=CH_3), warming with ethanol containing 5% of hydrochloric acid resulted in the formation of thiothiamine monohydrochloride of m. p. 221°, which differs in the melting point from the usually obtained salt of m. p. 243° but shows almost identical infrared absorption spectrum.

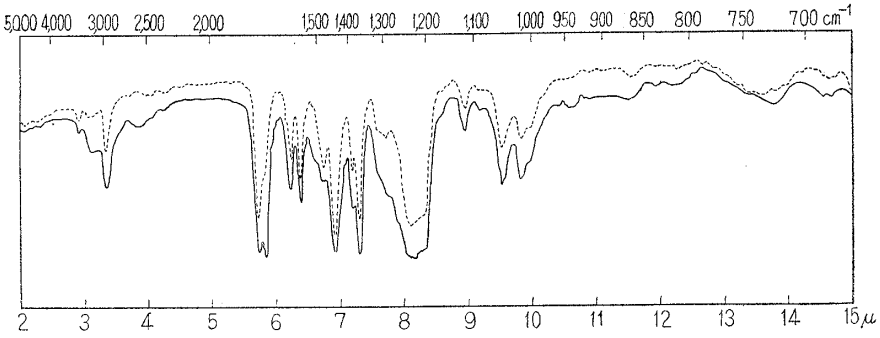
Saponification of O-acyl group alone by warming (V) with equivalent amount of ethanolic potassium hydroxide at 40~60°, by the Kunz method afforded N-acylthiothiamine (VII). In this case, however, a slight variation in reaction conditions results in further deacylation and the product contained (III).

Treatment of (V : R=CH_3) with a small amount of alkali or its recrystallization from

5) R. A. Abramovitch: J. Chem. Soc., **1957**, 1413.
6) S. Uyeo, S. Takagi: Yakugaku Zasshi, **73**, 339(1953).

water affords an isomer of m. p. 122~123°, which returns to the original substance on treatment with acetic acid. The ultraviolet absorption spectrum of this isomer is the same as that of the original compound but its infrared spectrum has absorptions at 1739 and 1681 cm^{-1} and amide absorption has shifted markedly to a longer wave-length region (Fig. 3). The

Fig. 3. Infrared Absorption Spectra (in $CHCl_3$)



——— N, O-Diacetylthiothiamine (V : R=CH_3)
- - - - N, O-Diacetylpseudothiothiamine

absorption of the isomer in chloroform solution also differs from that of the original substance. It is therefore assumed that this substance is not merely a polymorphic isomer but a stereoisomer arising from structural difference. This substance is designated as N,O-diacetylpseudothiothiamine.

Oxidation of (V) to obtain N-acylated thiamine (VIII) was attempted by various methods such as dropwise addition of hydrogen peroxide to suspension of (V) in water[7] or concurrent desulfurization and oxidation with Raney nickel in weak acidity,[6] but only thiamine ester was obtained.

Attempt was also made to prepare DL-*α*-lipoic anhydride, the starting material for the synthesis of N-lipoylthiothiamine as a derivative of lipothiamine (I), by heating DL-*α*-lipoic acid with acetic anhydride or by application of methoxyacetylene[8] to the acid, but in either case, a rubber-like substance was obtained. This substance showed absorptions for acid anhydride at 1825 and 1757 cm^{-1} in its infrared spectrum and was considered to be a polymer of *α*-lipoic anhydride. The preparation of N-lipoylthiothiamine had, therefore, to be abandoned for a time

The authors are grateful to Dr. L. J. Reed for the donation of a valuable sample of DL-*α*-lipoic acid needed for this work. They are also indebted to Mr. H. Shindo for infrared spectral measurements, and to Misses Furukawa and Ohtsuka for elemental analyses.

Experimental

3-(2-Methyl-4-acetamido-5-pyrimidylmethyl)-3a-methyl-perhydrofuro [2, 3-*d*] thiazole-2-thione (IV: R=CH_3)—A mixture of 5 g. of (II) and 50 cc. of Ac_2O was warmed on a water bath at 70~90° by which the crystals dissolved gradually to form a clear, colorless solution. After heating for 3 hrs., the solvent was distilled off under a reduced pressure and a white solid of m. p. 165~167° was obtained. Yield, almost quantitative. Recrystallization from dehyd. EtOH gave colorless rhomboprisms, m. p. 167~167.5. Analytical values are listed in Table IV.

The propionyl compound (IV : R=C_2H_5) and butyroyl compound (IV : R=C_3H_7) were also obtained by a similar procedure.

Table IV.

R in (IV)	m. p. (°C)	Formula	Calcd. (%)			Found (%)		
			C	H	N	C	H	N
CH_3	167~167.5	$C_{14}H_{18}O_2N_4S_2$	49.71	5.32	16.57	49.55	4.93	16.07
C_2H_5	149~150	$C_{15}H_{20}O_2N_4S_2$	50.84	5.68	15.92	50.34	5.53	16.18
C_3H_7	104.5~105.5	$C_{16}H_{22}O_2N_4S_2$	52.50	6.02	15.30	53.01	5.66	15.82

7) S. Yoshida, M. Unoki: Yakugaku Zasshi, **72**, 966(1952).
8) G. Eglinton, E. R. H. Jones, *et al.*: J. Chem. Soc., **1954**, 1860.

Acid Treatment of 3-(2-Methyl-4-acetamido-5-pyrimidylmethyl)-3a-methyl-perhydrofuro〔2,3-*d*〕-thiazole-2-thione (IV: R=CH_3)—ii) A mixture of 0.7 g. of (IV: R=CH_3) and 7 cc. of 10% HCl was warmed on a water bath at 50~55° for 15 mins., cooled, and neutralized with 10% NaOH by which needle crystals precipitated out. The crystals were collected by filtration and recrystallized from 70% EtOH to needles, m. p. 240°, undepressed on admixture with thiothiamine.

ii) A mixture of 1 g. of (IV: R=CH_3) and 10 cc. of 5% HCl was warmed at around 50° for ca. 30 mins., basified to weak alkalinity with 5% NaOH, and the crystalline precipitate so formed was recrystallized from 70% EtOH to crystals of m. p. 175°, undepressed on admixture with tetrahydrothiothiamine.

Treatment of 3-(2-Methyl-4-acetamido-5-pyrimidylmethyl)-3a-methyl-perhydrofuro〔2,3-*d*〕thiazole-2-thione with Ethanolic Hydrochloric Acid—A mixture of 1 g. of (IV: R=CH_3) and 20 cc. of EtOH containing 5% of HCl was warmed at around 60° for 1 hr., the solvent was distilled off under a reduced pressure, and the syrupy residue was allowed to stand with 3 cc. of water, from which needle crystals, m. p. 221°, were obtained. *Anal.* Calcd. for $C_{12}H_{16}ON_4S_2 \cdot HCl$: C, 43.31; H, 5.11; N, 16.84. Found: C, 43.17; H, 5.40; N, 16.45.

This substance of m. p. 221° was neutralized with 5% $NaHCO_3$ and crystals of m. p. 240° separated out. This substance showed no m.p. depression on admixture with thiothiamine.

3-(2-Methyl-4-acetamido-5-pyrimidylmethyl)-4-methyl-5-(2-acetoxyethyl)-4-thiazoline-2-thione (V: R=CH_3)—A mixture of 5 g. of (III) and 50 cc. of Ac_2O was warmed on a water bath at 90~100°, by which the crystals dissolved gradually. After heating for 3 hrs., the solvent was distilled off under a reduced pressure and the yellowish solid residue was recrystallized from dehyd. EtOH to prisms, m. p. 134~135°. Yield, almost quantitative. Analytical values are given in Table V.

The dipropionyl compound (V: R=C_2H_5) and dibutyroyl compound (V: R=C_3H_7) were obtained by similar treatment.

TABLE V.

R in (V)	m. p. (°C)	Formula	Calcd. (%) C	H	N	Found (%) C	H	N
CH_3	134~135	$C_{16}H_{20}O_3N_4S_2$	50.53	5.26	14.74	50.31	5.22	14.81
C_2H_5	132~133	$C_{18}H_{24}O_3N_4S_2$	52.94	5.88	13.73	52.82	5.69	13.90
C_3H_7	104~105	$C_{20}H_{28}O_3N_4S_2$	55.04	6.42	12.84	55.23	6.40	12.45

Treatment of 3-(2-Methyl-4-acetamido-5-pyrimidylmethyl)-4-methyl-5-(2-acetoxyethyl)-4-thiazoline-2-thione (V: R=CH_3) with Ethanolic KOH—A mixture of 1 g. of (V: R=CH_3) and 13 cc. of 1% ethanolic KOH was warmed in a water bath of around 40° for 1 hr., the solvent was evaporated under a reduced pressure, and a small amount of water was added to the syrupy residue. This solution was extracted with ether and the extract of dried over $CaCl_2$. Evaporation of ether left a semi-solid which solidified on addition was dehyd. EtOH and was recrystallized from EtOH to crystals of m. p. 118.5~119.5°. *Anal.* Calcd. for $C_{14}H_{18}O_2N_4S_2$: C, 49.51; H, 5.32; N, 16.56. Found: C, 49.15; H, 5.21; N, 16.38.

Similar treatment of the above product with 0.5% EtOH-KOH afforded crystals of m. p. 122~123°. *Anal.* Calcd. for $C_{16}H_{20}O_3N_4S_2$: C, 50.53; H, 5.26; N, 14.74. Found: C, 50.56; H, 5.37; N, 14.49.

This substance of m. p. 122~123° is also obtained on dissolving (V: R=CH_3) in hot water and allowing this solution to cool.

3-(2-Methyl-4-diacetylamino-5-pyrimidylmethyl)-4-methyl-5-(2-acetoxyethyl)-4-thiazoline-2-thione (VI: R=CH_3)—A mixture of 2 g. of (V: R=CH_3), 10 cc. of pyridine, and 5 cc. of Ac_2O was heated on a water bath for 1 hr., the mixture was allowed to stand over night at room temperature, and the solvent was evaporated under a reduced pressure. The syrupy residue was triturated with dehyd. EtOH and a white solid of m. p. 110° was obtained in almost quantitative yield. Recrystallization from dehyd. EtOH gave plate crystals, m. p. 113~115°. *Anal.* Calcd. for $C_{18}H_{22}O_4N_4S_2$: C, 51.18; H, 5.21; N, 13.27. Found: C, 51.62; H, 5.15; N, 13.76.

Summary

1) Acetylation of perhydrofurothiothiamine (II) and thiothiamine (III) with acid anhydride respectively gave N-acylated perhydrofurothiothiamine (IV) and N,O-diacylated thiothiamine (V). Treatment of N,O-diacetylthiothiamine with acetic anhydride in pyridine afforded N,N,O-triacetylthiothiamine (VI). Alkali treatment of (V) gave N-acylated thiothiamines.

2) Attempts to obtain lipoic anhydride, for the synthesis of N-lipoylthiothiamine, were without avail.

(Received May 16, 1958)