The authors express their deep thanks to Dr. H. Mima for his kind encouragement in these studies, and to Takeda Chemical Industries, Ltd. for permission to publish this report.

**Summary**

Infrared spectroscopic methods were applied for investigations on the states of molecules trapped in thiourea-\(\mathrm{d}_4\) channels. It was found that, in the spectra of thiourea-\(\mathrm{d}_4\) adducts, absorption bands due to guest molecules could be observed in the regions between 2800 and 2600 cm\(^{-1}\), 2200 and 1600 cm\(^{-1}\), 1300 and 990 cm\(^{-1}\) and 790 and 680 cm\(^{-1}\). Thus, nearly all absorption bands of guest molecules were observed in sodium chloride region by the use of both thiourea and thiourea-\(\mathrm{d}_4\) adducts, except the regions where CH\(_2\) stretching and bending vibrations absorb. As an example, bands due to the axial isomers of halogenated cyclohexanes were determined by this technique. Relatively free rotation or random orientation of dioxane or cyclohexane molecules in thiourea channels was also supported by the present work. From the comparison with the orientation of cyclpentanone or cyclohexanone molecule in thiourea channels, it is suggested that electric dipole forces would operate to orientate guest molecules in channels.

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42. Satoru Kuwada and Masatake Hori: Supplement of the Notice on the Formation of 6-Methyl-7-hydroxyribolumazine. (Application of Chromatography. XLVI.\(^*3\)).

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

In order to investigate unexamined ingredients of the mycelium of *Eremothecium ashbyii* by thin layer chromatography (TLC), the fresh mycelium of the microorganism was extracted with water at 80° for 15 minutes. The extract was concentrated at 45–50° in vacuo and an aqueous solution of the residue was applied on a thin layer of alumina or silica gel G and developed with such solvents as dimethylformamide or butanol series. As a result it was confirmed that separation was most effective in a combination of silica gel G and butanol–ethanol–water (50:15:35). Investigation of the chromatogram, however, revealed that while the spots of riboflavin (V. B\(_1\)) and 6-methyl-7-hydroxyribolumazine (V Comp.) were separated clearly, 6,7-dimethylribolumazine (G Comp.) was considerably tailed and the green fluorescence was very faint. Just when the authors were about to investigate the cause, Uehara\(^*3\) reported at the Vitamin Committee that the carbon atom (–CH–) at the position 2 and 8 of purine compounds such as hypoxanthine and adenine is converted to -CH by photochemical oxidation in the presence of V. B\(_1\), namely, uric acid is produced from hypoxanthine. It seemed that V Comp. was not originally present in the mycelium but produced from G Comp. in the

\(^*1\) Part XLV: This Bulletin, 11, 23 (1963).

\(^*2\) Juso-nishino-cho, Higashiyodogawa-ku, Osaka (桑田 哲, 滋 正剛).

course of concentration of the extract or during the chromatography by contact with light, because readily oxidizable G Comp. coexists with V. B₂ in the extract of *Er. ashyii*. The process of oxidation of G Comp. by enzyme is probably different from that of the oxidation of hypoxanthine, but the oxidative reaction in the presence of V. B₂ and light is considered as a general chemical reaction, so this process may be applied to the conversion of G Comp. to V Comp.

To ensure the assumption, two strains of V. B₂-producing *Er. ashyii*, (a) and (b), were newly incubated in a polypeptone medium with shaking. After about 70 hours the mycelium was filtered, washed with cold water repeatedly and immediately extracted with the same weight of water, in an atmosphere of nitrogen for 15 minutes at 80°, and then filtered with filter paper. On the other hand, the mycelium was extracted with water using a homoblenor cooling with ice water and the extract was filtered as above. Each of the two samples thus obtained was developed on a thin layer of silica gel G with butanol–ethanol–water (50:15:35) for about 2 hours at room temperature, avoiding sunlight. On the chromatograms G Comp. (Rf 0.35, green fluorescent), V Comp. (Rf 0.41, purple fluorescent) and V. B₂ (Rf 0.57, yellow fluorescent) were observed in all the samples derived from (a) and (b). Consequently it must be considered that V Comp. was not produced by the mechanism presumed by the authors but it was originally present in the mycelium, as G Comp. was. The authors⁹ have reported that there existed enzymes in the mycelium of *Er. ashyii*, which converted G Comp. to V. B₂ and V Comp. It is quite reasonable, therefore, that as the mycelium requires oxygen for its growth, the existence of V Comp. may be assumed if G Comp. and V. B₂ were present.

On the other hand, as there is V. B₂ in the extract, is there not the possibility of G Comp. changing into V Comp. by a long time exposure to scattering light during the concentration of the mycelium extract? To make this point clear, the extract (pH 6.05~6.23) was exposed to direct sunlight for 15 hours and investigated by TLC. As a result it was found that the green fluorescent spot at Rf 0.35 (G Comp.) disappeared or became very faint and blue fluorescent spots were detected at Rf 0.68 and 0.83 besides those of V Comp. (Rf 0.41) and V. B₂ (Rf 0.57) as shown in Fig. 1. In this case, as the determination of the purple fluorescent spot of V Comp. was difficult, it was merely observed with the naked eye to find that the intensity of the fluorescence was almost the same before and after the photochemical oxidation.

To identify the two spots at Rf 0.68 and 0.83, G Comp. was exposed to scattering light for 2 hours at a light window at pH 4.09~8.83 and then applied to TLC. The spot of G Comp. disappeared completely at pH 4.09~5.41 and a blue spot appeared at Rf 0.68, while at pH 6.52~8.83 the spot of G Comp. also disappeared and a purple and a blue spot appeared at Rf 0.41 and 0.68, respectively. The former was identified as V Comp. by comparing it with an authentic sample and also from the ultraviolet spectrum of its aqueous extract shown in Fig. 2. From its Rf value and from the color tone of its fluorescence the latter must be 6,7-dimethylumazaine (GM), that is, a photochemical oxidation product of G Comp. As formation of 6,7,8-trimethylumazaine (GM) from G Comp. was supposed in this case, GM was applied to TLC under the same conditions, when a green fluorescent spot appeared at Rf 0.25. But no GM was detected in the above-mentioned photochemical oxidation product of G Comp. Incidentally, the substance at Rf 0.83 was not obtained by photochemical oxidation of G Comp.

Conversion of G Comp. to V Comp. was hitherto effected by an enzymic method or by introduction of air into a solution of G Comp. in a dark place at a basic pH⁹ but

the present study observed that V Comp. is formed by merely exposing an aqueous solution of G Comp. to light at a pH higher than 6.52, concurrently with a substance produced by the elimination of the ribityl group at N₈ of G Comp., which may also be formed at a lower pH. It is known that V Comp. as such or at a basic pH is photochemically oxidized to 2,4,7-trihydroxy-6-methylpteridine (VH) in the presence of hydrogen peroxide. In this case, as formation of 6,8-dimethyl-7-hydroxylumazine (VM) was also presumed, the Rf values and the color tones of the fluorescences of VH and VM in TLC were investigated with the above photochemical oxidation product of G Comp., but none of them were detected. From the foregoing, it is understood that the photochemical oxidation of G Comp. to V Comp. does not proceed further unless more drastic conditions are applied. This may be due to the difference in the structures of the mother nucleuses of the two compounds.

When G Comp. was photochemically oxidized in the presence of V. B₃ at various pH's from 4.09 to 8.83, blue spots appeared at Rf 0.68 and 0.83 in all the cases, but at pH 6.52~8.83 an additional purple spot also appeared at Rf 0.41. From the above description it is evident that the spot at Rf 0.41 is V Comp. and that at Rf 0.68 is GH. What is then the blue fluorescent spot at Rf 0.83? To identify it, V. B₃ was photochemically oxidized at pH 4.09~8.83 and applied to TLC, and it was found that at pH 4.09~5.41 the yellow spot of V. B₃ almost disappeared and a blue spot appeared at Rf 0.83, and at pH 6.52~8.83 the yellow spot of V. B₃, the yellow spot of lumiflavine (Lum. F.) and the blue spot of lumichrom (Lum. C.) appeared at Rf 0.57, 0.70, and 0.83, respectively. This result coincided with that of Shimizu, which was obtained by photodecomposing V. B₃ at various pH's and developing the product on filter paper with butanol-acetic acid-water. Therefore, the spot at Rf 0.83 was identified as Lum. C.

From the results mentioned above the authors wish to conclude as follows:
1) The mycelium of *Er. ashyii* producing V. B₃ (yellow type strain) contains G and V Comps. besides V. B₃.
2) The aqueous extract of *Er. ashyii* is subject to oxidative decomposition during its concentration or other processes. As a result, Lum. F. and Lum. C. are produced from V. B₃ and GH from G Comp.
3) G Comp. is photochemically oxidized at any pH to produce GH, and at pH > 6.52 V Comp. is produced in addition to GH, but it is not further oxidized under the conditions described in this report.
4) When G Comp. is subjected to photochemical oxidation in the presence of V. B₃ under the same conditions as in 3), formation of Lum. C. due to V. B₃ and the products in 3) is clearly observed, but presence of Lum. F. is hardly evidenced probably for its small quantity.

**Experimental**

1) **Cultivation of *Er. ashyii***—Strain No. 5 (a) and the V. B₃ and FAD-producing strain (b) preserved in the Institute for Fermentation, Osaka, were subjected to slant culture in a peptone medium for 7 days and the seed cultures thus obtained were transferred into 500 ml. of the medium for main culture and cultivated further at 28° for 72 hr. under shaking. The pH of the medium was not adjusted all the time, but it was 6.8~7.0 during the seed culture and 6.0~6.2 during the main culture.

2) **Extraction of the Mycelium**—Each of the mycelia of (a) and (b) was filtered on filter paper with suction and washed 3~4 times with cold dist. H₂O until the washing became colorless to give 10.9~22.5 g. of wet mycelium.

An amount of 3~5 g. of the mycelium was warmed with the same volume of H₂O at 80° for 15 min. in an atmosphere of N₈, filtered on Toyo filter paper No. 50 with suction and the filtrate was used.

as a sample. On the other hand, the mycelium was extracted with about two times its weight of dist. H$_2$O with ice cooling, using a homoblendor of Nihon Seiki Co. The blender was operated at low speed at first for 3 min. and then at high speed (about 20,000 r.p.m.) for 20 sec., and the extract, after being filtered as above, was used as another sample.

3) **Investigation by Thin Layer Chromatography**—A slurry of Merck’s silica gel G was applied on a glass plate, 20 × 20 cm., with the applicator of Desaga Co. to make a thin layer of 0.25 mm. and dried at 105°C for about 3 hr. Each of the samples was applied on the thin layer and developed with BuOH-EtOH-H$_2$O (50:15:35), and the chromatogram was observed under UV rays.

i) Extracts of the mycelium: In all of the two kinds each of the two extracts of (a) and (b) there were clearly observed the spots of G Comp. (Rf 0.35, green fluorescent), V Comp. (Rf 0.41, purple fluorescent), V.B$_2$ (Rf 0.57, yellow fluorescent) and some unidentified yellow spots.

ii) Photodecomposition of the mycelium extract: An amount of about 0.3 ml. of the mycelium extract i) was exposed to direct sunlight for 1.5 hr. in a small test tube. The extract thus treated and the same extract but not exposed to sunlight were applied on the starting line of a TLC plate together with ca. 0.3% aqueous solutions of G and V Comp., GM, GH, VM, and VH at intervals of 2 cm. and developed with BuOH-EtOH-H$_2$O (50:15:35). When the solvent front reached 10 cm. after 2 hr., the plate was lifted from the solvent and, after evaporation of the solvent, observed under UV rays to give the chromatogram shown in Fig. 1.

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**Fig. 1.**

As is evident from Fig. 1 the spot corresponding to G Comp. disappeared completely and the fluorescence of V Comp. did not intensify. Instead, a blue fluorescent spot (Rf 0.68) appeared at a Rf larger than that of V.B$_2$ (Rf 0.57, yellow fluorescent) and another blue fluorescent spot at Rf 0.83.

The blue spot at Rf 0.68 is obvious to be GH from Fig. 1 and from the photodecomposition products of pure G Comp., while the blue spot at Rf 0.83 is evidenced to be Lum. C. from the result of photodecomposition of V.B$_2$.
The GM, GH and VH used as control substances were synthesized previously and their structures were established, and VM was newly synthesized. In the synthesis of VM, 2,4,6-trichloropyrimidine (I) was used as the starting material and I→V was conducted according to the method of Masuda\(^8\) and V→VM according to the methods of Masuda, Kishi and Asai\(^9\) and Masuda, Kishi, Asai and Kuwada\(^8\) as shown in the above chart.

The synthetic VM (VM) was a yellow crystalline substance m.p. >300\(^\circ\) (decomp.) from 2\(N\) HCl emitting a strong greenish purple fluorescence in the aqueous solution, and its N value (25.76%) was roughly in accord with that (26.91%) calculated from C\(_9\)H\(_{18}\)N\(_2\)O\(_2\). As the UV spectrum of this substance showed a gentle curve having its maximum around 280 m\(\mu\) as shown in Fig. 2, it is clearly different from V Comp. and VH. When the substance was developed on filter paper with BuOH-EtOH-H\(_2\)O (50:15:30), its Rf value was 0.20 and it was 0.42 when developed on a thin layer of Merck’s silica gel G (0.39 with silica gel H). The substance migrated +3.0 cm. when subjected to paper electrophoresis in Theorell buffer of pH 7.0 (pH = 0.1, 300 v. 4 hr.). From these results it seems reasonable to think that this lumazine compound takes the structure of 6,8-dimethyl-7-hydroxy-3(8\(H\))-pteridinedione (VM).

iii) Photochemical oxidation of G Comp.: About 1 mg. of G Comp. was dissolved as such and together with the same amount of V.B\(_2\) in 0.3 ml of phosphate buffer of various pH's (4.09 to 8.83) and the solutions were placed at a light window for ca. 2 hr., protecting from direct sunlight and then investigated by TLC to give the results shown in Fig. 3. When G Comp. was used alone, the same chromatograms were obtained at any pH lower than 5.41, while chromatograms at pH higher than 6.52 were all nearly the same, but the higher pH the more intense the purple fluorescence of V Comp. This purple fluorescence should be of V Comp. because when the photochemically oxidized sample of G Comp. was applied on the above-mentioned thin layer in a large quantity and developed under the same conditions as before and the portion of the purple fluorescent spot was extracted with H\(_2\)O, the extract showed the same UV absorption as an authentic V Comp. (cf. Fig. 2). At any rate, the fluorescent spot of G Comp. disappeared completely and GH was formed at any pH, while at pH >6.52 increase of V Comp. was observed. Fig. 4 is the chromatogram obtained in the coexistence of G Comp. and V.B\(_2\), where G Comp. was not detected at any pH, and besides the spot of the remaining V.B\(_2\), the blue fluorescent spots of GH and Lum. C. were observed at Rf 0.68 and 0.83, respectively. And at pH's higher than 6.52 the purple fluorescent spot of V Comp. appeared in addition to the above products.

iv) Photodecomposition of V.B\(_2\): V.B\(_2\) was dissolved in phosphate buffer of various pH's (4.09~8.83) to prepare about 0.3% solutions, which were then placed by a window for one day on a cloudy day and investigated by TLC to give the result shown in Fig. 5. According to a report of Shimizu\(^8\)

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8) T. Masuda: This Bulletin, 5, 28 (1957).
the Rf of the photodecomposition products of V. B₀ is almost the same whether developed with an acidic solvent or with a neutral solvent. In the present study the spots of Lum. F, and Lum. C, were identified from their Rf values and fluorescence colors described in the report of Shimizu because the samples were not at hand. And the yellow spot at Rf 0.57 was regarded as the remaining V. B₀, the yellow spot at Rf 0.70 as Lum. F, and the blue spot at Rf 0.83 as Lum. C. Among the spots that of Lum. C was appeared in the photodecomposition product at any pH but that of Lum. F. was observed only in the products at a pH higher than 6.52. This result also is in good agreement with that of Shimizu.

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Summary

To determine whether the V Comp. detected in the extract of Er. ashbyi was present originally in it or produced supplementally by photooxidation of G Comp., the mycelium was extracted in an atmosphere of nitrogen and the extract was investigated by TLC. The result proved the original existence of all of V. B₀, G and V Comps. But taking into consideration Uehara's report on hypoxanthine. G Comp. was examined if it is photooxidized to V Comp. Namely, the extract of the mycelium was exposed to sunlight and investigated by TLC, when GH and Lum. C were detected apparently but V Comp. did not increased. On the other hand, G Comp. was photodecomposed in phosphate buffer at various pHs, when GH was produced at any pH, while V Comp. was formed only at pH > 6.52 besides GH. The same experiment was conducted in the presence of V. B₀, when Lum. C was produced at any pH, but Lum. F. at pH > 6.52 in addition to the above products.

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43. Ken Ikeda and Hajime Takeda: Reaction between Salicylic Acid and Zinc Oxide in the Presence of Water.

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Zinc oxide is compounded with salicylic acid in various dermatological preparations such as lotion, ointment, and paste. It has been known that various unexpected phenomena occur in such preparations. Kitchin¹ and Anon² observed the appearance and solidification of a lotion containing zinc oxide and salicylic acid. Kitchin reported that the change did not occur for two or three days after preparation of the lotion that when it did occur on the fourth day, the change was very rapid. They concluded that such change was due to the formation of basic zinc salicylate, but did not ascertain its formation of chemically. Meanwhile, Strakosch³ observed that keratolytic action of salicylic acid was inhibited when it was compounded with zinc paste. In recent years,

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