obtained by the chromic oxidation of MHB, were completely identical and the two absorption bands at 5.96 μ ($\nu_{C=O}$) and 6.19 μ ($\nu_{C=C}$) suggested the existence of α,β-unsaturated ketone in their structure.

MHB-M (Ⅲ), which was also isolated from the urine of rabbits receiving MHB, possessed a considerably sharp band at 2.87 μ and this suggested the existence of a hydroxyl group in its structure.

The authors are indebted to Sankyo Co. Ltd. for the measurement of the infrared sbsorption spectra and also to Dainippon Seiyaku Co. Ltd. for their supply of materials.

Summary

The ultraviolet absorption spectra of the metabolites from the urine of rabbits receiving MHB, the oxidation products of MHB and nor-MHB with chromium trioxide were measured, and the relation between these compounds and their structures was discussed.

The infrared absorption spectra of 3-keto-MHB and MHB-M (Ⅱ) from the previous work in this series indicated that the two are completely identical and would be α,β-unsaturated ketones and that of 3-OH-MHB suggested the existence of a hydroxyl group in the structure.

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U. D. C. 547.854.4'861.2 : 582.284

74. Toru Masuda : Application of Chromatography. XXIX.[1] G Compound isolated from the Mycelium of *Eremothecium ashbyii*.

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

The components of the mycelium collected at various stages of the culture of *Eremothecium ashbyii* were studied by paper partition chromatography, and a green (G compound) and a purple (V compound) fluorescent bands were detected on the chromatograms of samples collected at a comparatively early stage. The former, which was already reported in a communication,[2] attracted a keen interest of the author because it seemed to have a close relation with the formation of riboflavin in the mycelium.

In the present paper the extraction, isolation, and purification of the substance, which were touched on in the communication, are described in more detail and further discussion is made on its structure.

Experimental

Isolation of G Compound—1) Two hundred grams of the wet mycelium obtained by 80-hr. culture of *Er. ashbyii* was extracted 3 times with a 500 cc. portion of water at 80° for 15 mins. and the combined extract was concentrated to 500 cc. The separated riboflavin was removed, 250 g. of $(NH_4)_2SO_4$ was added to the solution, and the resulting brown substance was filtered off. The filtrate was shaken 3 times with a 30-cc. portion of phenol and the phenol extract was shaken again with 500 cc. of ether and 30 cc. of water to transfer all flavine compounds into the water layer. The last operation was repeated twice and the combined aqueous solution was subjected to ad-

* Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (増田 亨).

1) This constitutes a part of a series entitled "Application of Chromatography" by Satoru Kuwada. Part XXVIII. This Bulletin **3**, 434 (1955).

2) T. Masuda : This Bulletin **4**, 71 (1956).

sorption with Florisil, as shown in the next paragraph, or they were extracted with the same volume of benzyl alcohol, the extract was shaken with ether and water, and the aqueous solution, after being concentrated, was subjected to column chromatography on powdered cellulose.

2) **Separation by Florisil**—Fifty grams of Florisil (60 mesh) was packed into a glass tube (diam., 2.5 cm.) and the column was irrigated with 50 cc. of the above-mentioned aqueous solution containing 5 cc. of AcOH. The resulting effluent was non-fluorescent because riboflavin and G compound had been adsorbed. Next, the column was washed with 100 cc. of 5% AcOH, when a brown impurity was eluted. The column was again irrigated with 5% pyridine solution, whereupon G compound was eluted in the first ca. 50 cc. of the effluent, which was recognized by disappearance of the green fluorescence from the column. Riboflavin was eluted in the last place. The G-compound fraction was concentrated *in vacuo*, dissolved in 20 cc. of 50% EtOH, and the solution was allowed to stand at room temperature, when a brown impurity precipitated. The impurity was filtered off, the filtrate was evaporated, and the residue was recrystallized from 80% EtOH to yellow crystals.

3) **Purification with Powdered Cellulose Column**—One hundred and fifty grams of powdered cellulose was impregnated with water saturated with benzyl alcohol and packed into a glass tube (diam., 4 cm.). A dough consisting of 4 cc. of the above-mentioned aqueous solution and 12 g. of powdered cellulose was placed thereon, and the column was irrigated with benzyl alcohol in a dark room, whereupon a brown impurity, riboflavin, a light yellow impurity, and G compound eluted in 6~20 cc., 20~85 cc., 85~230 cc., and 230~350 cc. fractions, respectively. The last fraction was shaken with twice its volume of ether and 30 cc. of water to transfer G compound into the aqueous layer and the aqueous solution was evaporated to dryness *in vacuo*, leaving a yellow residue, which was recrystallized from 80% EtOH to light yellow crystals. Yield, ca. 15 mg. from 200 g. of the wet mycelium.

Properties of G Compound—G compound, m.p. 273~274°(deccomp.); $[\alpha]_D^{20}$ −164°. It is readily soluble in water, sparingly soluble in MeOH and EtOH, but insoluble in ether, $CHCl_3$, and benzene. Judging from its band in paper electrophoresis, this substance seems to be a neutral or slightly basic substance. It is negative to S test with nitroprusside, halogen test with $AgNO_3$, and P test with ammonium molybdate, but positive to N test. To detect the amino group, the substance was diazotized and, after addition of NaN_2, subjected to azotometry, but the reaction was negative. Moreover, it was negative to color reactions with *p*-dimethylaminobenzaldehyde and with diazotized sulfanilic acid. *Anal.*(dried at 130° for 3 hrs.) Calcd. for $C_{13}H_{18}O_6N_4 \cdot \frac{2}{3}C_2H_5OH$: C. 48.22; H, 6.17; N, 15.69; C_2H_5OH, 8.60. Found : C, 48.18; H. 6.32; N, 15.54; C_2H_5OH, 8.34. *Anal.* (the above sample recrystallized from water and dried at 130° for 3 hrs.) Calcd. for $C_{13}H_{18}O_6N_4$: C, 47.85; H, 5.56; N, 17.17; mol. wt. 326. Found : C, 48.20; H, 5.65; N, 17.01; mol. wt. (Barger), 284 ±30 (H_2O).

Ultraviolet Spectra (Fig. 1)—Measurement was made on solutions of 1.54 mg. of G compound in 50 cc. of the solvents.

Water and acid solutions : λ_{max} 258 and 407 mμ.
Alkaline solution : λ_{max} 280 and 315 mμ.

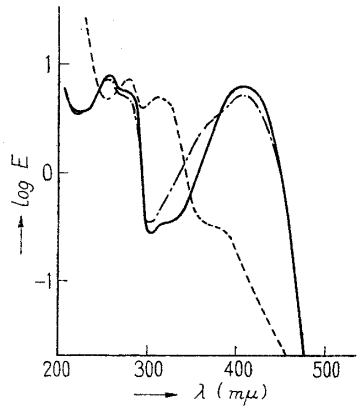


Fig. 1.

——— in H_2O
—·—·— in 0.1 *N* HCl
---------- in 0.1 *N* NaOH

Infrared Spectra (Fig. 2)

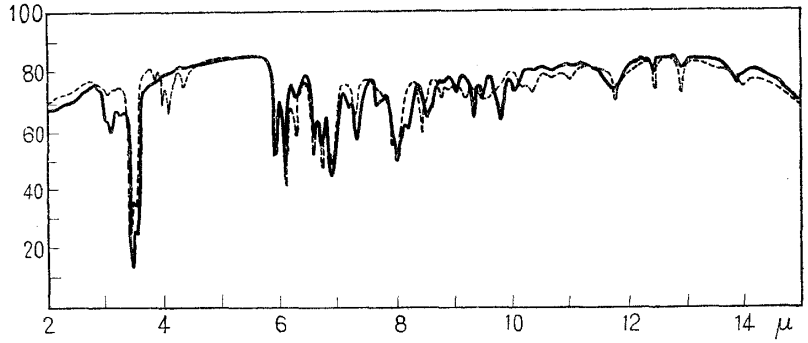


Fig. 2.

——— G compound
····· Deuterated G compound

Change of G Compound by $Na_2S_2O_4$—Aqueous solution of the substance loses its fluorescence by the addition of $Na_2S_2O_4$ but regains it by oxidation with air. This phenomenon is the same as in the case of riboflavin.

Decomposition by Hot Alkali Solution—Ten milligrams of the substance was heated with 0.1 cc. of 1*N* NaOH at 100° for 30 mins. The mixture was developed on paper with EtOH•BuOH•H_2O (15:50:35) and the chromatogram was observed under ultraviolet rays or after spraying with EtOH *p*-dimethylaminobenzaldehyde solution, when two green fluorescent bands with Rf 0.17 and 0.37, and a band with Rf 0.4, positive to the Ehlrich reagent, were detected. The band with Rf 0.4 corresponds to urea. This band was always found on chromatograms of the alkali decomposition products of riboflavin and lumiflavin as shown in Table I.

Table I.

Sample	Rf and color of the spot				*p*-Dimethylaminobenzaldehyde Reactn.
G Compound	0.17G+	0.37G			0.40+
Riboflavin		0.34Y+	0.49GY	0.59B+	0.40++
Lumiflavin	0.20Y	0.34Y	0.52B		0.40+
Urea					0.40++

G: green, Y: yellow, B: blue fluorescence
+, ++, +++: Intensity of the coloring

Photochemical Decomposition Product (Formation of 6,7-Dimethyllumazine)—When a solution of 30 mg. of G compound in 2 cc. of water in an ampule was exposed to direct sunlight for 10 days, the green fluorescence disappeared and crystals deposited, which were recrystallized from water into colorless needles (ca. 15 mg.), m.p. 340~345°(decomp.)(uncorr.).

This substance is readily soluble in MeOH, EtOH, and hot water but insoluble in ether and $CHCl_3$. When developed on paper with BuOH•EtOH•H_2O, it gives a single blue fluorescent band with Rf 0.53. In addition, this substance gives the same Rf values as synthetic 6,7-dimethyllumazine when developed with various solvent systems (Table II) and a mixture of both substances always gives a single band. Like the substance before decomposition the product is negative to S, halogen, P, and NH_2 tests, but positive to N test. *Anal.* Calcd. for $C_8H_8O_2N_4$: C, 49.99; H, 4.20; N, 29.16. Found: C, 49.77; H, 4.40; N, 28.86.

Table II.

Solvent System	G-Compd. photolysate	6,7-Dimethyllumazine
BuOH•EtOH•H_2O (50:15:35)	0.53	0.53
BuOH•AcOH•H_2O (4:1:5)	0.56	0.56
BuOH•pyridine•H_2O (4:4:7)	0.70	0.70
Benzyl alcohol•H_2O	0.60	0.60

Ultraviolet Spectrum (Fig. 3)—The solid line shows the curve in a neutral solution, in which the absorption maxima appear at 250 and 330 mμ. The dotted line shows the curve in an alkaline solution, in which the absorption maxima appear at 240, 272, and 350 mμ.

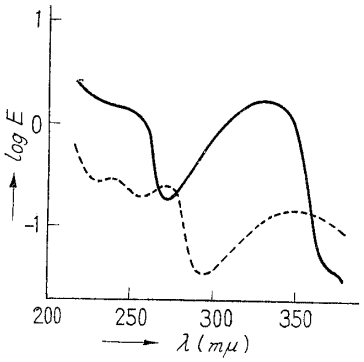


Fig. 3.
——— in H_2O
---------- in NaOH

Infrared Spectra (Fig. 4)—The solid line shows the photochemically decomposed product of G compound and the dotted line, the same sample recrystallized from D_2O.

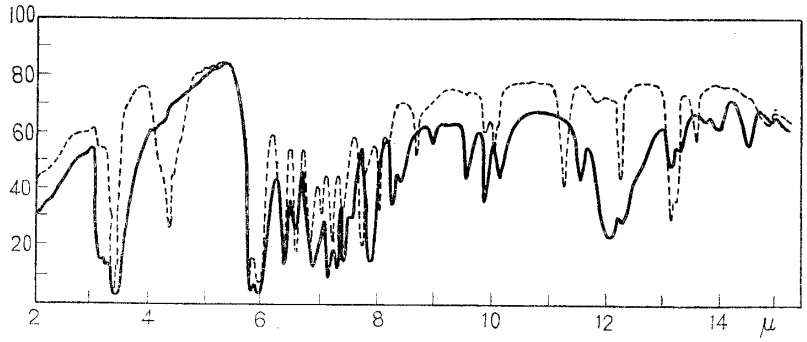


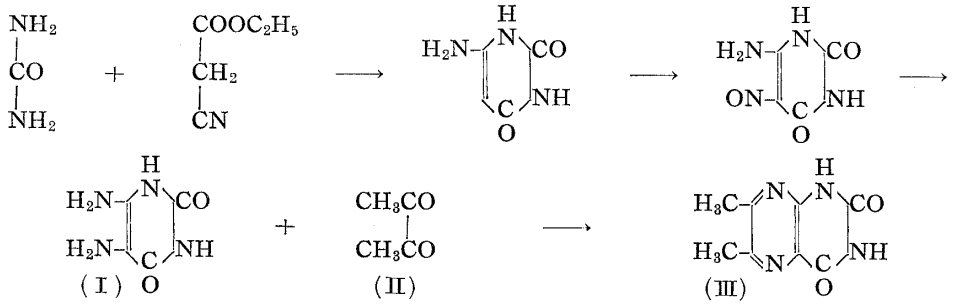
Fig. 4.

Detection of Polyhydric Alcohol—The mother liquor from the above needle-like crystals (6,7-dimethyllumazine) was developed with the solvent systems shown in Table III. The bands detected with ammonia-alkaline $AgNO_3$ solution and alkaline $KMnO_4$ solution seemed to be due to a polyhydric alcohol. The result was in good accord with the result obtained as follows: An aqueous solution of riboflavin acidified with AcOH was decomposed photochemically, lumichrome and lumiflavin were removed with $CHCl_3$, the aqueous solution was concentrated *in vacuo*, and subjected to paper chromatography.

Table III.

Sample \ Solvent System	BuOH•EtOH•H_2O (4:1:5)	BuOH•pyridine•H_2O (4:3:7)
From G compd.	0.45‖ 0.55+	0.69+
From riboflavin	0.45‖ 0.55±	0.69+

Synthesis of 6,7-Dimethyllumazine—4,5-Diamionuracil (I), prepared from urea and ethyl cyanoacetate according to the method of Bogert and Davidson,[4] was reacted with diacetyl (II) in water according to the methods of Kuhn and Cook,[5] and of Weijlard and Tishler.[6] The product was recrystallized twice from water to colorless needles, m.p. 348°(decomp.)(uncorr.). *Anal.* Calcd. for $C_8H_8O_2N_4$: C, 49.99; H, 4.20. Found: C, 49.97; H, 4.37.



4) M. T. Bogert, D. Davidson: J. Am. Chem. Soc., **55**, 1667(1933).
5) R. Kuhn, A. H. Cook: Ber., **70**, 761(1937).
6) J. Weijlard, M. Tishler, A. E. Erickson: J. Am. Chem. Soc., **67**, 802(1945).

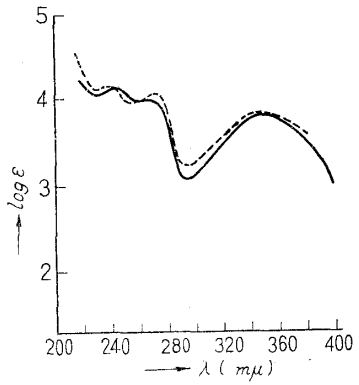


Fig. 5. Ultraviolet Spectra
——— 6,7-Dimethyllumazine
····· G compd. photolysate in NaOH

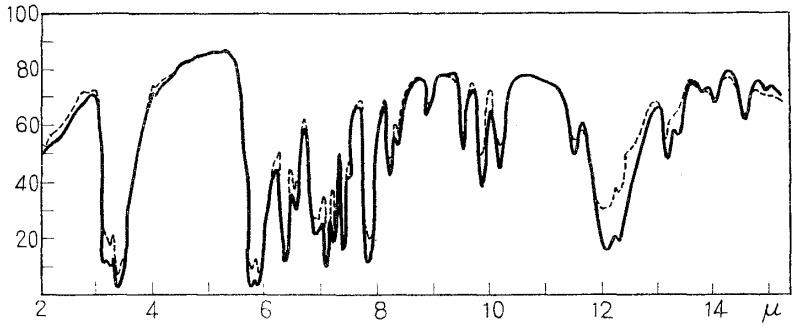


Fig. 6. Infrared Spectra
——— 6,7-Dimethyllumazine
····· G compd. photolysate

Discussion

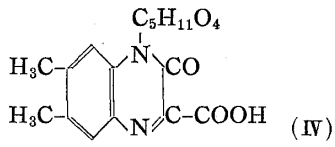
In the previous paper,[2] the composition of G compound was reported as $C_{14}H_{18}O_7N_4$. Subsequent study revealed, however, that the substance contained hardly removable ethanol. So it was again analyzed after recrystallization from water and thorough drying to give the values of $C_{13}H_{18}O_6N_4$. The molecular weight measured by the Barger method was roughly in accord with this composition.

The infrared spectrum of the substance was reported in the previous paper and in the present work, change of the infrared spectrum by deuteration was observed. As already reported by Kanzawa and Masuda this method furnishes important informations for elucidating the structure of compounds of this series. As seen in Fig. 2, the complicated absorptions near 3 μ migrated to the vicinity of 4 μ, and a slight change was also observed around 6 μ where ring vibration and NH-deformation vibration are generally reflected. Further, a marked change was found in the region of 9~10 μ, where one of the groups susceptible to deuteration is a hydroxyl. The absorptions near 3 μ imply the existence of some OH or NH groups with hydrogen bonds of different strength. In general, the spectrum of sugars appears in a wide range covering 9~10 μ. Therefore, if one of the OH groups of a sugar is converted into OD, a change must be observed in this region. The above spectral change by deuteration and the fact that G compound has fairly strong levorotatory power are enough to suggest that the compound has a polyhydric alcohol moiety.

The ultraviolet spectrum of G compound was also reported in the previous paper. In the present work the spectra of the compound in 0.1*N* HCl and 0.1*N* NaOH were compared. As is evident from Fig. 1, the change of the spectrum by change of pH is different from that of riboflavin and the fact seems to indicate that the parent structure of G compound is different from that of riboflavin.

G Compound gives polarographic reduction waves at pH 1~12, and the relation between the reduction waves and pH is represented by the equation $E^{1/2} = -0.26 \sim 0.058$ pH. The wave height of the first step gradually decreases as pH lowers from 6, and

at the same time the second step appears at a more negative potential, but the total wave height is constant between pH 1 and 9. This behavior of the compound is different from that of riboflavin or 1-ribityl-2-oxo-6,7-dimethylquinoxalinyl-3-carboxylic acid (IV), which has a quinoxaline ring and rather resembles the behavior of the compounds of pteridine series. When the molecular weight of the compound is taken as 326, its diffusion current constant is 2.55 μ AmM^{-} mg$^{-2/3}$t^{-3} sec$^{1/2}$. This value resembles 2.49 of riboflavin, whose reduction wave involves two-electron process. Hence the reduction of G compound seems to be participated by two electrons, and at higher pH than 6, it is assumed to take a structure with different reducibility.

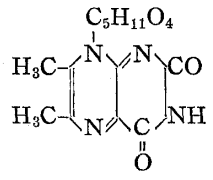


(IV)

The photochemical decomposition product of G compound is helpful in clarifying the structure of this compound. When an aqueous solution of the compound was exposed to direct sunlight, it lost the green fluorescence and needle-like crytals deposited. The product, m.p. 340~345°(decomp.), was more soluble in organic solvents than the parent compound, and when developed on paper with butanol-ethanol-water it showed Rf 0.53 as against Rf 0.22 of G compound. The analytical value of the product corresponded to $C_8H_8O_2N_4$, which is in accord with the value of G compound minus $C_5H_{10}O_4$. If the above decomposition was effected in the same manner as in the formation of lumichrome by the photochemical decomposition of riboflavin, $C_5H_{10}O_4$ must be a ribityl group.

As seen from Figs. 5 and 6, the ultraviolet (Fig. 3) and infrared spectra (Fig. 4) of the product are in good accord with those of synthetic 6,7-dimethyllumazine. In addition, both substances always showed the same Rf value when developed separately or together with various solvent systems.

Assuming that G compound has a ribityl group it is not yet clear what would be produced if the ribityl is split off by the photochemical decomposition of G compound. However, when a neutral solution of G compound and a solution of riboflavin in 5% acetic acid (as riboflavin is sparingly soluble in water, it was dissolved in dilute acetic acid) were subjected to photochemical decomposition as before and then developed with BuOH•EtOH•H_2O (4 : 1 : 5) and with BuOH•pyridine•H_2O (4 : 3 : 7), two bands responding to alkaline silver nitrate solution as well as to alkaline potassium permanganate solution were detected with the former solvent and one band with the latter solvent. Though the bands are not yet fully identified, the above supports the fact that the band derived from G compound is identical with that derived from riboflavin.



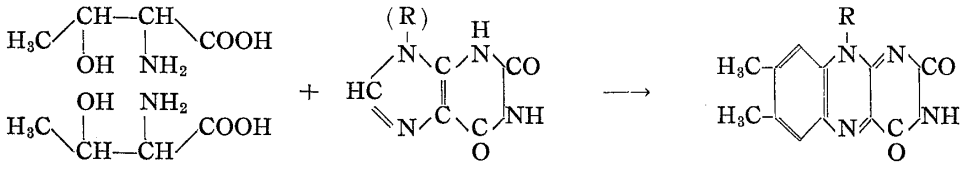
Although no definite evidences have been obtained as yet, it is obvious that the photochemical decomposition product of G compound is 6,7-dimethyllumazine, and in view of the fact that the polyhydric decomposition product derived from G compound resembles that derived from riboflavin, the structure shown might be assigned to G compound.

Previously, McLaren[7] reported that the production of riboflavin in the culture of *Er. ashbyii* was increased by the addition of adenine, guanine, or xanthine, but decreased by the addition of uracil. Duplicating the experiment, McNutt[8] stated that free purine componds are more favorable for the formation of riboflavin than nucleosides and nucleotides. In the experiment he also investigated transition of purine ring to flavin ring. That is, he conducted the culture after addition of adenine whose C_8 or another carbon atom had been replaced with ^{14}C, and investigated the radioactivity of the

7) J. A. McLaren : J. Becteriol., **63**, 233(1952).
8) W. S. McNutt : J. Biol. Chem., **210**, 511(1954).

resulting riboflavin and its photochemical decomposition product, lumichrome. As a result it was found that when adenine whose carbon atoms other than C_8 had been replaced by an isotope was used, about 80% of radioactivity was detected in the isoalloxane ring, whereas when adenosine whose C_8 had been replaced by the isotope was employed almost no radioactivity was detected in the resulting riboflavin.

Goodwin and Pendlington[9] also studied conditions for the formation of riboflavin in the culture of *Er. ashbyii* and found that *l*-threonine and *l*-serine are favorable for the formation of riboflavin, but that *l*-tyrosine is not so favorable as the former, and that other amino acids rather inhibit the formation. Further, they found that purine, xanthine, and adenosine are conducive to the formation of flavine ring, but 5,6-dimethylbenzimidazole and 1,2-dimethyl-4-amino-5-(D-1′-ribitylamino)benzene have no relation with the formation. From the results they represented the mechanism for biosynthesis of riboflavin as follows :

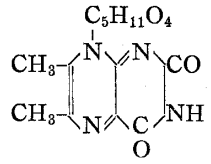


It is not yet thoroughly clear whether G compound is an intermediate for the biosynthesis of riboflavin or an abnormal metabolite, but as a mere hypothesis it may be allowed to suppose that G compound is synthesized from acetic acid or glycine and a purine compound according to the idea of Goodwin *et al.* and further develops into an isoalloxane derivative.

The author wishes to express his appreciation to Mr. Hideo Kamio and to Mr. Tokunosuke Kanzawa for the measurement and analysis of infrared spectrum, to Mr. Yutaka Asahi for the measurement of polarography, and to the members in charge of elementary analysis. Thanks are due also to Mr. Yoichi Sawa and Mrs. Mitsuko Asai for their cooperation throughout the work.

Summary

Detailed explanation was made on the isolation of G compound from the mycelium of *Er. ashbyii*, and its purification. The composition of this compound was established as $C_{13}H_{18}O_5N_4$. A key compound for elucidating the structure of G compound was isolated as yellow needles, m.p. 340~345° (decomp.), from the photochemical decomposition products of the latter, and it was identified as 6,7-dimethyllumazine from its Rf value and ultraviolet and infrared spectra. From these facts and from the analysis of the infrared spectrum of G compound, the structure shown was assigned to the compound.



(Received June 13 1956)

9) T. W. Goodwin, S. Pendlington : Biochem. J. (London), **57**, 631(1954).