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A Naturally Occurring Indolylpteridine*

Walter S. McNutt and Isao Takeda

ABSTRACT: A mutant strain of *Achromobacter petrophilum* produces a green-yellow fluorescent compound when grown in a medium containing an excess of guanine. The efficient incorporation of ^{14}C from [2-^{14}C]guanine (but not from [8-^{14}C]guanine) into the unknown substance suggested that it might be a pteridine. The substance, crystallized from 4 N HCl, gave dark red needles with a neutralization equivalent of 465 and the analysis $C_{19}H_{21}N_5O_7 \cdot 3H_2O$. It consumed 3 moles of periodate/mole of compound and was converted upon reduction with Zn and HCl into a blue fluorescent compound and indole. The blue fluorescent compound was characterized as 8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine. The structure of the green-yellow fluorescent compound was established as 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine by chemical synthesis. The substance differs from naturally occurring pteridines hitherto known in having an indolyl group on the 6 position of the pteridine ring.

Takeda and Hayakawa (1968) obtained a purine-requiring mutant of *Achromobacter petrophilum*, which produces a green-yellow fluorescent compound when cultured in the presence of excess guanine.

In this article evidence is presented to show that Takeda's green-yellow fluorescent compound is another in the class of naturally occurring ribityllumazines (Masuda, 1956; Masuda *et al.*, 1958; McNutt, 1960). It differs from the naturally occurring pteridines previously known in having a 3-indolyl group at position 6 of the pteridine. Its structure has been established as 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (I).

Results

The ^{14}C of [2-^{14}C]guanine, but not [8-^{14}C]guanine, was effectively incorporated into the green-yellow fluorescent compound by cultures of the organism (Figure 1), suggesting that the compound was probably a pteridine.

The neutralization equivalent (465; see Experimental Section) showed the presence of a large group or groups on the pteridine ring, and the periodate consumption; 3 moles of periodate/mole of compound (see Experimental Section) was consistent with a ribityl group.

The substance analyzed, $C_{19}H_{21}N_5O_7 \cdot 3H_2O$, and as hitherto known ribitylpteridines are lumazines it seemed likely that one of the nitrogen atoms was in a side chain. Also, the ultraviolet absorption spectrum showed a marked bathochromic shift in 4 N HCl (Figure 2, B *vs.* C) unlike that expected of a 2-amino-4-hydroxypteridine, and this indicated a weakly basic nitrogen atom in the side chain in conjugation with the pteridine ring.

The substance was readily reduced by Zn and HCl, giving rise to a blue fluorescent compound having ultraviolet absorption spectra (Figure 3) similar to those of 6-methyl-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (III) (McNutt, 1960). The structure of the blue fluorescent compound was established as 8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine (II) by

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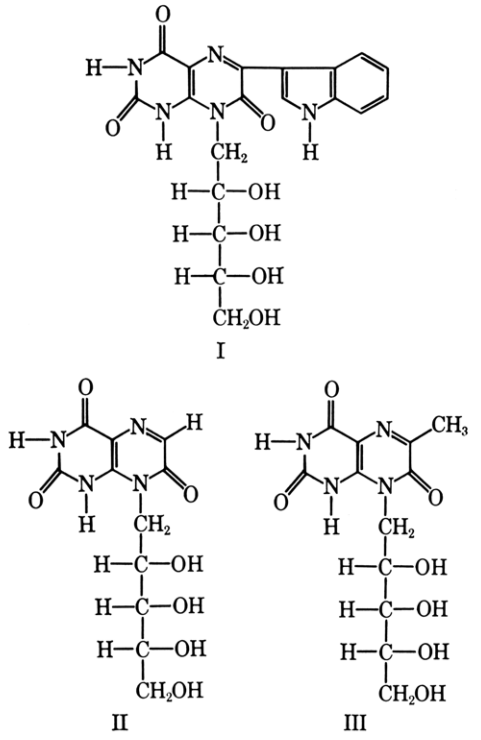

I

II

III

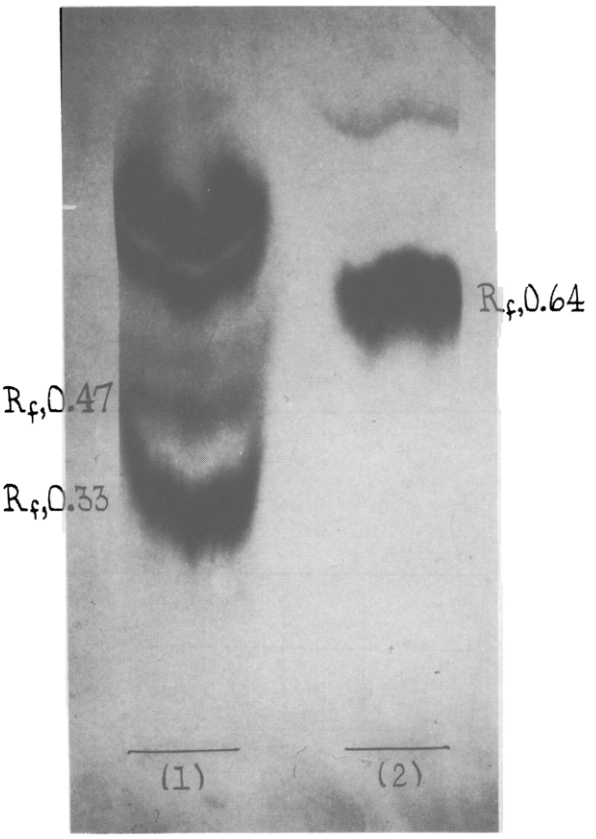

FIGURE 1: Incorporation of [2-^{14}C]guanine into the green-yellow fluorescent compound. (1) The autoradiogram of fluorescent compounds separated on paper in water saturated with benzyl alcohol (solvent front, 24 cm). In this system the green-yellow fluorescent compound (R_F 0.33) was well separated from other fluorescent substances. Perfect correspondence as to position and shape was obtained between the fluorescence and the radioactivity of this band. Incorporation of ^{14}C into a blue fluorescent compound (R_F 0.47) and other fluorescent substances is shown. (2) The corresponding autoradiogram of an amount of [2-^{14}C]guanine (R_F 0.64) equivalent to that added in the fermentation. Note the efficient incorporation of [2-^{14}C]-guanine into fluorescent compounds by this organism.

chemical synthesis of this substance and comparison of it with the blue fluorescent compound (Figure 4).

The aromatic amine arising from Zn–HCl reduction of the green-yellow fluorescent compound gave less than 8% of the value expected for a primary aromatic amine in the Bratton–Marshall (1939) test. It was characterized as indole (see Experimental Section) following its isolation from impurities in the steam distillate (Figure 5). The strong color given by the green-yellow fluorescent compound in the Folin–Ciocalteau test (McNutt, 1964) is probably due to the indolyl group, as III is negative in this test whereas indole gives a positive test.

As the green-yellow fluorescent compound is stable to acid, it was reduced in an attempt to render the side chain of the reduced pteridine more susceptible to hydrolysis. However, the products of Zn–HCl reduction suggest actual hydrogenolysis of the C—C bond between the 6 position of the pteridine and the 3 position of indole. The susceptibility of this C—C bond between the two aromatic rings to cleavage by hydrogenolysis seems reasonable in view of the ease with which hydrogenolysis of the C—C bond of pentaphenylethane occurs (Adkins and Shriner, 1953).

The 3-indolyl rather than the 2-indolyl linkage to the 6 position of the pteridine seemed likely in view of its probable biosynthetic relationship with tryptophan. Also, the 3-indolyl linkage has the cation of indole in conjugation with the pteridine ring, and this agreed with the observed bathochromic shift in 4 N HCl (Figure 2, B *vs.* C).

Chemical synthesis of I and comparison of the synthetic and natural substances established the structure of the green-yellow fluorescent compound as 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine.

Discussion

It is not evident what function this substance might have in the metabolism of this organism. The ribityllumazines previously known to be elaborated in considerable amount by microorganisms have been associated with the production of large quantities of riboflavin, whereas the *Achromobacter* from which this compound has been isolated does not similarly produce the vitamin in excessive amount.

It seems likely that a metabolic derivative of tryptophan or a tryptophan precursor serves as the intermediate in the biosynthesis of this compound. Indolylglyoxylic acid itself or its "biological equivalent" is

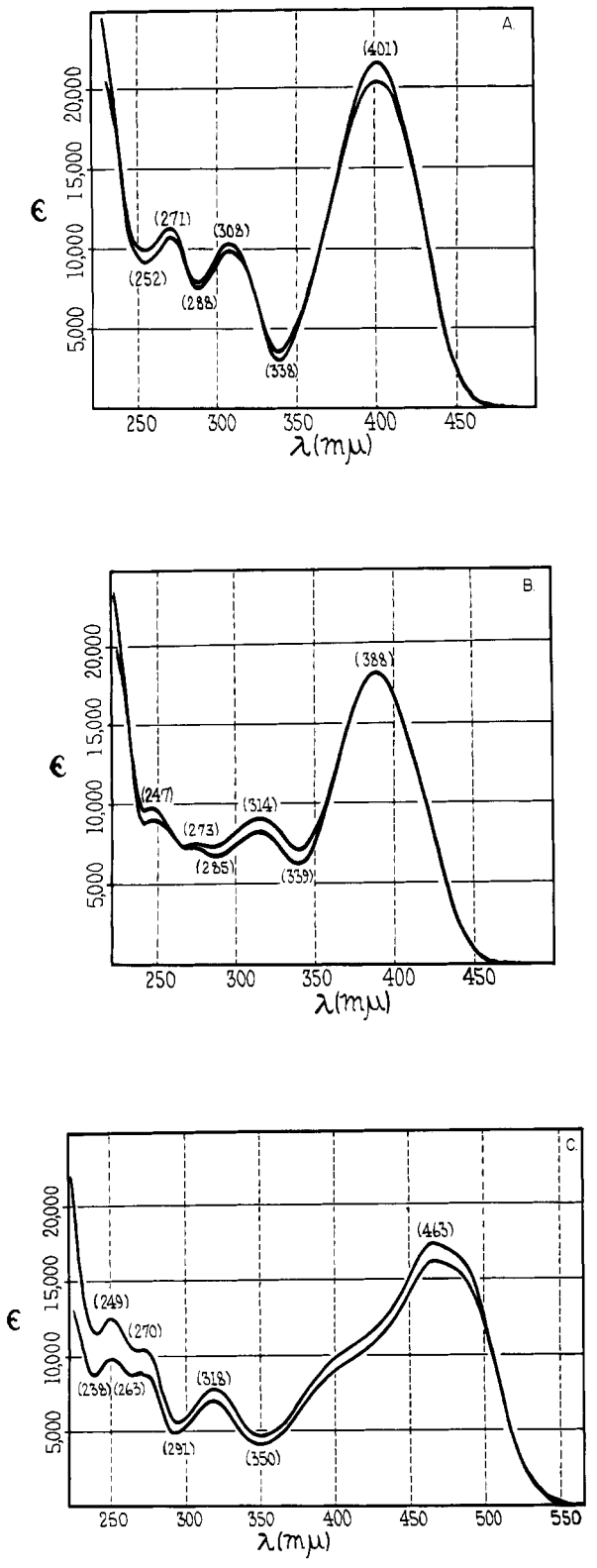

FIGURE 2: The ultraviolet absorption spectra of the green-yellow fluorescent compound. (A) In 0.1 N NaOH, (B) in 0.1 N HCl, and (C) in 4 N HCl. The upper curve is the molecular extinction coefficient of 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine prepared chemically. The lower curve is that of the natural compound. The values in parentheses are the maxima and minima of the natural compound.

apparently not known as a constituent of the cell (Meister, 1965).

Completion of the pyrazine ring of pteridines from carbon atoms of the side chain of tryptophan would represent an alternate metabolic pathway of pteridine biogenesis. Biosynthetic studies carried out with ring- and side-chain-labeled tryptophan could be readily interpreted, for the hydrogenolysis reaction with Zn–HCl affords an easy way to measure the distribution of label between the indole and pteridine rings.

As for the possible significance of 6-indolylpteridines, one might note that enzymes capable of hydroxylating simple pteridines at position 6 have not yet been found, whereas enzymatic hydration (Meister, 1965, p 847) of a 6-indolylpteridine, should it occur, would provide a mechanism for introducing an oxygen atom at this position.

Experimental Section

Achromobacter Fermentation. A. petrophilum No. 4510 (Takeda and Hayakawa, 1968) was cultured in the following medium: 1 l. contained *n*-hexadecane (Eastman Co.), 50 ml; KH_2PO_4, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 4.0 g; Difco yeast extract, 6.0 g; $(NH_4)_2SO_4$, 4.0 g; hypoxanthine, 10 mg; and guanine, 100 mg. The pH was adjusted to 6.8–7.0 with KOH. $CaCO_3$ (20 g) was added and the volume was adjusted to 1 l.

Portions of this medium (50 ml/250-ml ehrlenmeyer flask) were inoculated from the stock culture and incubated at 30° on a mechanical shaker for 4 days. Pooled cultures (300 ml) grown in this way were used to inoculate 6 l. of medium in a 14-l. jar fermentor, and the culture was grown under forced aeration at 30°. Maximum production of the green-yellow fluorescent compound usually occurred after 3–4 days.

Isolation of the Green-Yellow Fluorescent Compound. Ammonium sulfate (3.6 kg) was added to 6 l. of a 3-day-old culture, and precipitated proteins and cells were filtered. To the filtrate phenol (600 ml) was added, whereupon the green-yellow fluorescent compound passed into the phenol layer (Crammer, 1948). The phenol layer was separated and mixed well with ether (600 ml) and water (180 ml), whereupon the green-yellow fluorescent compound passed into the aqueous phase. After evaporation of the ether the aqueous phase was passed through a 2.5 × 30 cm column of DEAE-cellulose (in the form of the free base). By washing the column with 1 l. of water and with 500 ml of 0.3% $(NH_4)HCO_3$ solution other fluorescent compounds were eluted while leaving on the column the green-yellow fluorescent compound. It was subsequently eluted with 1 l. of 2% $(NH_4)HCO_3$ solution. The $(NH_4)HCO_3$ was removed by evaporation under vacuum, and the residue was dissolved in water. The solution was treated with 3 g of HCl-washed Norit. The Norit was washed with water and treated with 300 ml of 30% aqueous pyridine solution. The pyridine was removed from the eluate by evaporation under vacuum, and the solution (15 ml) was again chromatographed on a DEAE-cellulose column (1.5 × 25 cm)

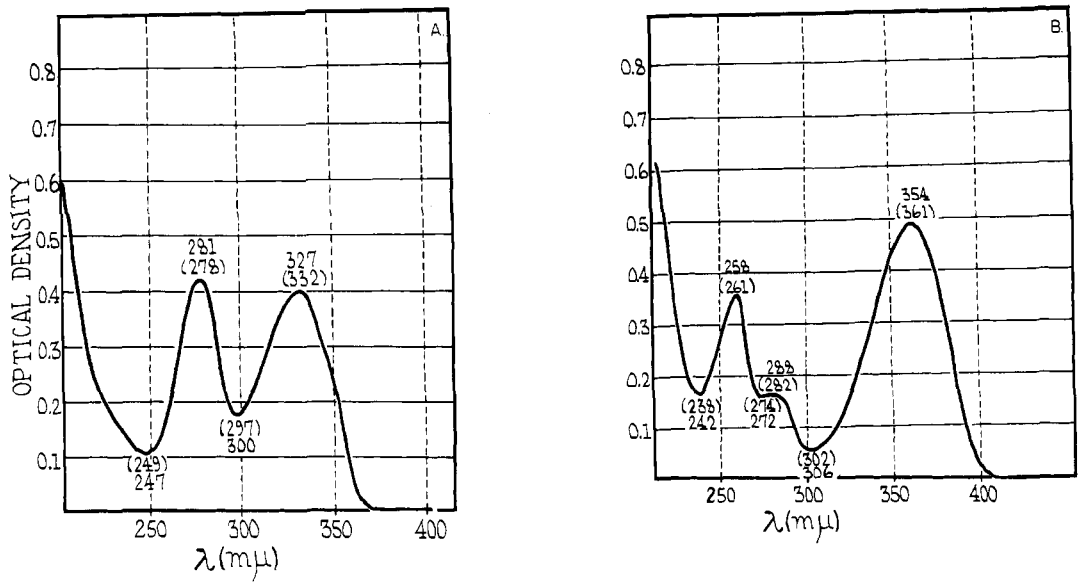

FIGURE 3: The ultraviolet absorption spectra of the blue fluorescent compound. (A) In 0.1 N HCl and (B) in 0.1 N NaOH. The numbers in parentheses are the values for the maxima and minima of the blue fluorescent compound. The numbers not enclosed in parentheses are the corresponding values for III.

which was subsequently washed with 1 l. of water and with 300 ml of 0.3% $(NH_4)HCO_3$ solution. The green-yellow fluorescent compound was eluted with 600 ml of 2% $(NH_4)HCO_3$ solution. The residue from the evaporated eluate was dissolved in 15 ml of water and adjusted to pH 1.0 with 1 N HCl. Yellow acicular crystals separated.

The $(NH_4)_2SO_4$ precipitate, from the first step, was washed with 500 ml of ether and extracted with 50% aqueous acetone solution made 0.1% with respect to NH_3. The extract was evaporated to 100 ml under vacuum, centrifuged, and the clear supernatant solution was worked up as above. In this way a combined total of 52 mg of red crystals was obtained from 6 l. of culture. Considerable variation was encountered in the yield of compound from different fermentations.

The substance crystallized well from hot 4 N HCl as dark red hair-like crystals. The crystals in the dry state were either dark red or yellow depending upon how well they had been washed with water, even though they were dried over NaOH under vacuum, mp 297–299° dec (melting point stage).

The Incorporation of [2-^{14}C]Guanine into the Green-Yellow Fluorescent Compound. To 20 ml of culture medium, as described above but with the guanine omitted, [2-^{14}C]guanine (Schwarz; 3.32 μCi/mg; 10 μCi) was added. The medium in a 125-ml ehrlenmeyer flask was inoculated and cultured aerobically at 30° for 88 hr. An aliquot (15 ml) of this culture was centrifuged, and the clear supernatant solution was adjusted to pH 6.0 and treated with 400 mg of Norit. The Norit was washed with 0.05 N HCl and eluted with 70 ml of 30% aqueous pyridine solution. After evaporation of the eluate to a small volume approximately one-third of it was placed along 3–4 cm of Whatman No. 1 filter paper, and the chromatogram was developed (Figure 1). Autoradiography showed

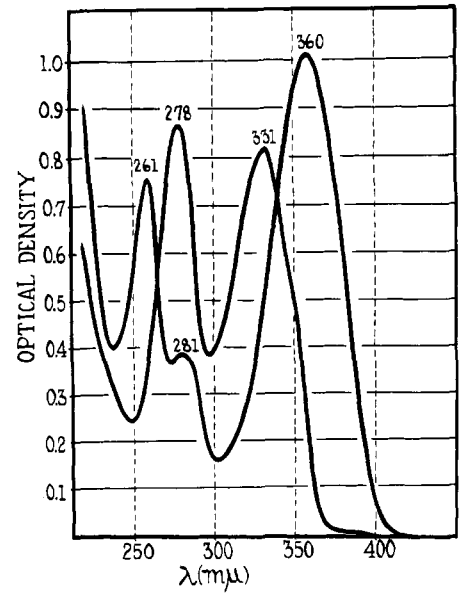

FIGURE 4: The ultraviolet absorption spectra of 8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine. The curve having three maxima is the absorption curve in 0.1 N NaOH, and the curve having two maxima is that obtained in 0.1 N NCl.

perfect matching of the green-yellow fluorescent compound (R_F 0.33; Figure 1) and radioactivity. Also, efficient incorporation of [2-^{14}C]guanine into fluorescent compounds by this organism is shown.

A comparable experiment with [8-^{14}C]guanine (Schwarz; 3.30 μCi/mg; 10 μCi) showed no clearly discernable incorporation of ^{14}C into the green-yellow fluorescent compound or the other fluorescent substances.

Neutralization Equivalent. Prior to titration the

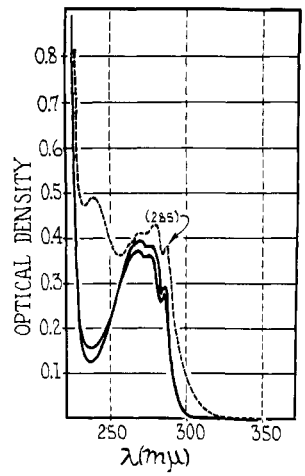

FIGURE 5: The absorption spectrum of the aromatic amine derived from the green-yellow fluorescent compound. The spectrum of the steam distillate prior to removal of impurities is represented by the dashed curve. The upper solid line is the absorption spectrum of indole in water (8.25 μg/ml). The lower solid line is the absorption spectrum of the steam distillate after impurities had been removed (see text). That impurities still remained is evident from the fact that the steam distillate while having a lower absorbancy than the indole standard at the maxima showed a greater absorbancy than the indole standard in the region of the minimum.

compound was recrystallized from hot 4 N HCl, washed with water, and dried over solid NaOH at room temperature and 30 mm. Dark red crystals were obtained. Crystals (6.00 mg) were dissolved in 30 ml of hot water. A stream of N_2 was bubbled through the cooled solution while titrating with 0.00945 N NaOH; 1.364 ml was required: duplicate 5.97 mg, required 1.358 ml. *Anal.* Calcd for $C_{19}H_{19}N_5O_7 \cdot 3H_2O$: neut equiv 483. Found: 465, 465; 3.7% error. pK_a = 4.75.

Periodate Titration. To a solution of 5.37 mg of green-yellow fluorescent compound in 10 ml of 0.002 N NaOH, saturated $NaHCO_3$ solution (2.00 ml) and 0.200 M $NaIO_4$ (0.400 ml) were added and the reaction was allowed to proceed at 24°. At the times indicated aliquots (2.00 or 2.50 ml) were withdrawn and titrated for residual periodate (Jackson, 1944). The results shown in Table I are calculated on the basis of a molecular weight of 483. *Anal.* Calcd for $C_{19}H_{19}N_5O_7$: C, 53.14; H, 4.46; N, 16.31; O, 26.08. Found in material dried to constant weight at 100° and high vacuum: C, 53.04, H, 4.83; N, 16.11; O, 25.81. Calcd for $C_{19}H_{19}N_5O_7 \cdot 3H_2O$: water of crystallization, 11.18%. Found: the specimen dried in a desiccator at room temperature and barometric pressure lost 11.36% of its weight upon drying to constant weight at 100° and high vacuum; *O*-methyl: Found: 0.00%.

TABLE I: Periodate Oxidation of the Green-Yellow Fluorescent Compound.

Time (hr)	Moles of Periodate Consumed/ Mole of Compd
0.5	1.65
1.0	2.05
2.0	2.81
3.5	3.07
19.5	3.67

Absorption Characteristics of the Green-Yellow Fluorescent Compound. The molecular extinction coefficient of the substance is shown in Figure 2.

Proof That the Pteridine Product of Zn–HCl Reduction is 8-(1-D-Ribityl)-2,4,7-trioxohexahydropteridine. A. CHARACTERISTICS OF THE PRODUCT. The green-yellow fluorescent compound (3.2 mg; 6.63 μmoles) in 3 ml of 2 N HCl was dissolved by warming, and to the cooled solution a small amount of zinc dust was added to bring about complete decolorization. After standing overnight the solution was placed along 102 cm of Whatman No. 3MM filter paper, whereupon a pink color appeared upon drying. The chromatogram (40-cm excursion) was developed in isopropyl alcohol–concentrated HCl–water (120:41:93, v/v) (Wyatt, 1951). In this system the R_F values of the green-yellow fluorescent compound and III were 0.15 and 0.31, respectively. Found on the chromatogram: principal product, blue fluorescent band, R_F 0.22; faint green-yellow fluorescent band, R_F 0.07; and faint reddish bronze fluorescent band, R_F 0.57.

The areas of paper containing the blue fluorescent compound (R_F 0.22) were quantitatively eluted with water, the eluate was evaporated under vacuum to a small volume, and the solution was rechromatographed in 1-butanol–glacial ascetic acid–water (120:30:50, v/v). The chromatogram showed a strong blue fluorescent band, R_F 0.17, and only trace amounts of two other blue fluorescent bands of higher R_F value. The ultraviolet absorption spectra (Figure 3) of the substance eluted from the paper with water (an eluate from a corresponding area of blank chromatogram served as the solution in the reference cell) resemble those of III (McNutt, 1960). The yield, calculated on the assumption that the blue fluorescent compound and III have the same molecular extinction coefficients at their absorption maxima in 0.1 N HCl, was 6.52 μmoles from 6.63 μmoles of green-yellow fluorescent compound. These values for III in 0.1 N HCl ((281 mμ (ε 11,530) and 327 mμ (ε 10,930)) are not very different from those of 8-methyl-2,4,7-trioxohexahydropteridine of pH 1.5 (278 mμ (ε *ca.* 10,000) and 330 mμ (ε 9900)) (Pfleiderer, 1957).

B. SYNTHESIS OF 8-(1-D-RIBITYL)-2,4,7-TRIOXOHEXAHYDROPTERIDINE. 1. 6-(1-D-Ribitylamino)-2,4-dioxypyrimidine (Maley and Plaut, 1959; McNutt, 1960). 6-Chloro-2,4-dioxypyrimidine (1.46 g), 70% D-ribamine (3.8 g), and water (7 ml) were heated in a sealed tube at 120–125° for 5.5 hr. The solution was taken up in water, treated with a little charcoal, filtered, and evaporated to dryness at 55°.

To the residue, from which 6-(1-D-ribitylamino)-2,4-dioxypyrimidine began crystallizing, methanol (50 ml)

was added, and the mixture was placed in the refrigerator overnight. The crystals were filtered, washed with methanol and with ether, and dried. The product (1.6 g) softened at 148°. The material recrystallized twice from water–methanol and melted at 182–185° (McNutt, 1960). *Anal.* Calcd for $C_9H_{14}N_3O_6$: C, 41.5; H, 5.4; N, 16.1. Found: C, 40.9; H, 5.7; N, 15.8.

2. Sodium 5-Nitroso-6-(1-D-ribitylamino)-2,4-dioxypyrimidine. 6-(1-D-Ribitylamino)-2,4-dioxypyrimidine (400 mg) was dissolved in 4 ml of warm water. Sodium nitrite (275 mg) and glacial acetic acid (5 drops) were added to the solution. A deep red-purple color developed, and red-orange crystals of monosodium 5-nitroso-6-(1-D-ribitylamino)-2,4-dioxypyrimidine separated. The crystals were washed with a small volume of cold water and with methanol, yield 380 mg. Material recrystallized from hot water–methanol, mp 167–170° (McNutt, 1960). *Anal.* Cacld for $C_9H_{13}N_4O_7{\cdot}Na$: C, 34.62; H, 4.19; N, 18.04. Found: C, 31.20; H, 4.46; N, 18.47.

3. 8-(1-D-Ribityl)-2,4,7-trioxohexahydropteridine. To sodium 5-nitroso-6-ribitylamino-2,4-dioxypyrimidine (25 mg; 80 μmoles) in 0.5 ml of water, sodium hydrosulfite (35 mg) was added and the deep red solution became pale yellow. Glacial acetic acid (15 μl) and glyoxylic acid (12 mg; 160 μmoles) were added and the solution (in a covered centrifuge tube) was heated at 90° for 10 hr. An aliquot of the reaction mixture (one-fifth of the total) was placed along 48 cm of Whatman No. 3MM filter paper. The chromatogram, developed in the butanol–pyridine–water system as described below, showed a main blue fluorescent product (R_F 0.21) and only small amounts of other fluorescent products; reference compound (the blue fluorescent product derived from Takeda's compound), R_F 0.22. The band (R_F 0.21) was eluted with dilute NH_4OH, and 0.5 ml of the evaporated solution (3.3 ml) was placed along 10 cm of Whatman No. 3MM filter paper. The chromatogram, developed in the butanol–acetic acid–water system, showed a major band, R_F 0.17 (reference spot R_F 0.17), and only trace amounts of other fluorescent substances. The 8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine was eluted from the paper and estimated spectrophotometrically, as described above. Found: 29 μmoles from 80 μmoles of sodium 5-nitroso-6-ribitylamino-2,6-dioxypyrimidine.

C. IDENTITY OF THE BLUE FLUORESCENT PTERIDINE AND 8-(1-D-RIBITYL)-2,4,7-TRIOXOHEXAHYDROPTERIDINE. (1) Chemically synthesized 8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine and the blue fluorescent pteridine derived from Takeda's compound agreed in their ultraviolet absorption characteristics in 0.1 N NaOH and in 0.1 N HCl (Figures 3 and 4). (2) The two substances cochromatographed as a single spot in six solvent systems (Whatman No. 3MM filter paper; 40 cm front; ascending method) as follows: 5% aqueous $(NH_4)HCO_3$), R_F 0.71; *n*-propyl alcohol–water–concentrated NH_4OH (80:40:2, v/v), R_F 0.35; *n*-butyl alcohol–pyridine–water (60:40:30, v/v), R_F 0.26; isopropyl alcohol–concentrated HCl–water (170:41:93, v/v), R_F 0.23; *n*-butyl alcohol–water–glacial acetic acid (120:50:30, v/v), R_F 0.14; and *t*-amyl alcohol–98% formic acid–water (65:30:5, v/v), R_F 0.07.

TABLE II: Absence of a Primary Amino Group in the Aromatic Amine.

Time	% of Theory (Sulfanilamide Equiv)
Immediately following decolorization	8.1
15 min later	3.4
After standing overnight	1.7

Negative Bratton–Marshall Test. The green-yellow fluorescent compound (446 μg) in 0.5 ml of 4 N HCl was reduced with a few milligrams of Zn dust. Aliquots (0.1 ml) were tested for the presence of a primary aromatic amine (Bratton and Marshall, 1939) and the results are shown in Table II.

Characterization of Indole. The green-yellow fluorescent compound (3.1 mg; 6.42 μmoles) was dissolved in 3 ml of 2 N HCl by warming. A small amount of zinc dust was added to the cooled solution. An amine-like odor was noted. As soon as the solution had become colorless, water (50 ml) was added, and the solution was neutralized with $NaHCO_3$, whereupon a faint pink color appeared. The solution was boiled, and the first 26 ml of distillate was collected. This steam distillate possessed a characteristic indole odor, gave a positive Ehrlich test (Allsop, 1941) (the color was somewhat more reddish than the red-violet color of indole), and showed the absorption maximum at 285 mμ characteristic of indole (Figure 5). The amount of indole recovered from 6.42 μmoles of compound (estimated from the optical density at 285 mμ) was 2.44 μmoles. The impurities in the steam distillate, presumably reduced products of indole, were removed by extraction with acid. The steam distillate (22 ml) was made 0.2 N HCl (a pale pink color appeared) and extracted with 40 ml of ether. The ether layer was washed with 20 ml of 0.2 N HCl, 10 ml of water, and 10 ml of 1% $NaHCO_3$ solution. Water (50 ml) was added to the ether layer, and the ether was evaporated. The aqueous solution was boiled, the first 10.5 ml containing 0.84 μmole of indole (estimated spectrophotometrically) and the next 11.5 ml containing 0.40 μmole (1.24 μmoles of indole thus recovered from 6.42 μmoles of compound). The distillate showed the ultraviolet absorption spectrum characteristic on indole (Figure 5). This distillate and a solution containing the same concentration of indole (that is, a solution having the same optical density of 285 mμ) responded similarly in the *p*-dimethylaminobenzaldehyde test (Allsop, 1941), in the 1,2-naphthoquinone-4-sulfonic acid test (Hawk *et al.*, 1951), in the nitroferricyanide test (Feigl, 1956), and in the colormetric test with xanthydrol (Dickman and Crockett, 1956) provided glacial acetic acid were used in the reagent and in the dilution. When concentrated HCl was used, the steam distillate gave only a very feeble color in comparison with the indole standard. The reason for

this discrepancy has not been examined further.

Pine Splint Test. About 0.2 mg of the green-yellow fluorescent compound was dissolved in 2 drops of 4 N HCl and zinc dust was added. As soon as the solution had become colorless it was subjected to the pine splint test (Sidgwick, 1945). It gave a red color which turned to purple upon drying, as indole did.

Folin–Ciocaltean Test. At the 1-μmole level (McNutt, 1964) the green-yellow fluorescent compound gave an intense blue color immediately. In this test III gave no color, whereas indole gave an immediate blue color.

Proof that the Green-Yellow Fluorescent Compound is 6-(3-Indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine. A. SYNTHESIS OF 6-(3-INDOLYL)-8-(1-D-RIBITYL)-2,4,7-TRIOXOHEXAHYDROPTERIDINE. To a solution of sodium 5-nitroso-6-(1-D-ribitylamino)-2,4-dioxypyrimidine (312 mg; 1 mmole) in 6.5 ml of water, sodium hydrosulfite (440 mg) was added followed by 0.2 ml of glacial acetic acid. 3-Indolylglyoxylic acid (mp 215–218° dec, lit. mp (Shaw *et al.*, 1958) 218° dec; 190 mg; 1 mmole) was added in portions with slight warning. After all had dissolved the solution was heated at 90° for 4 hr. The golden yellow mixture, which set to a gel on cooling, was diluted with 50 ml of hot water. A little charcoal was added, the solution was filtered, and the charcoal was washed with water. The resulting solution (70 ml) was warmed, and 1 ml of 5 N HCl was added. Upon cooling pale yellow crystals of 6-(3-indolyl)-8-(1-D-ribityl)-2,4,7-trioxohexahydropteridine separated. The crystals were washed with water and dried over NaOH under vacuum; 223 mg of orange-red product was obtained. For analysis 75 mg was dissolved in 80 ml of warm 5 N HCl, and the solution was filtered and diluted with 80 ml of hot water. Upon cooling microscopic yellow needles separated. The crystals were washed with a little water and dried over solid NaOH, yield 71 mg. *Anal.* Found in material dried at 100° under high vacuum: C, 53.05; H, 4.92; N, 16.09. Calcd for $C_{19}H_{19}N_5O_7$: C, 53.14; H, 4.46; N, 16.31.

B. THE IDENTITY OF THE GREEN-YELLOW FLUORESCENT COMPOUND AND 6-(3-INDOLYL)-8-(1-D-RIBITYL)-2,4,7-TRIOXOHEXAHYDROPTERIDINE. (1) The natural and synthetic substances agreed with regard to their ultraviolet absorption characteristics in 0.1 N NaOH, 0.1 N HCl, and 4 N HCl (Figure 2). (2) The synthetic and natural substances cochromatographed as a single spot in seven solvent systems. Specimens of synthetic and natural compound, as the free acid, were dissolved in NH_4OH and placed on Whatman No. 3MM. The two substances cochromatographed as follows: phenol (90% phenol in water)–4 N HCl (150:7, v/v), R_F 0.68; *n*-propyl alcohol–water–NH_4OH system, R_F 0.49; 1-butanol–pyridine–water system, R_F 0.36; methanol–water–morpholine (60:20:10, v/v), R_F 0.33; 1-butanol–water–acetic acid system, R_F 0.24; isopropyl alcohol–concentrated HCl–water system, R_F 0.15; and *t*-amyl alcohol–formic acid–water system, R_F 0.08. (3) On a melting point stage the acid form of the synthetic substance melted to a red resin and decomposed at 296–300°. The mixture melting point with the natural substance (297–299° dec) was 296–299° dec.

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