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The Structure of a Yellow Pigment from the Mutant *lemon* of *Bombyx mori*

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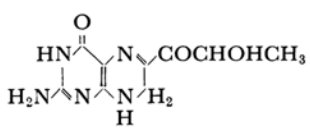
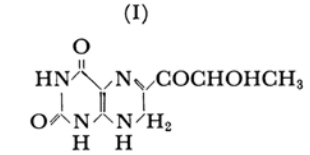
The structure of N_8-methylsepiapterin was previously postulated for a new pteridine from *Bombyx mori lemon* (This Bulletin, **38**, 503 (1965)). This structure must, however, be modified on the basis of some new experiments. We will now present the 7, 8-dihydro-6-lactyllumazine structure for this compound.

Hirata, Nakanishi and Kikkawa[1] have reported the isolation of a yellow pigment, named xanthopterin-B, from the epidermis of the mutant *lemon* of *Bombyx mori*. Nawa and Taira[2] have reported that xanthopterin-B is indistinguishable from a yellow compound found in comparatively large quantities in the eyes of the *sepia* mutant of *Drosophila melanogaster*. Aruga, Kawase and Akino[3] have shown, by chromatographic techniques, the presence of two yellow, yellow-fluorescent compounds (xanthopterin-B_1 and xanthopterin-B_2) in the epidermis of *B. mori lem.* Tsusue and Akino[4] have succeeded in isolating a small amount of xanthopterin-B_1, which is by far the major component, in a pure state and have demonstrated that it has the same structure as sepiapterin, i. e., 2-amino-4-hydroxy-7, 8-dihydro-6-lactylpteridine I.[5] The second component, xanthopterin-B_2, is present in extremely small amounts, and it is very sensitive to light. In a preliminary report,[6] we described some degradations of this compound and postulated a structure for it. This structure must, however, be modified in the light of some new experiments which will be described here. We now wish to propose the structure II for this compound.



(I)



(II)

Experimental

The Isolation of Xanthopterin-B_2.—The epidermis of silkworms (*B. mori*, mutant *lemon*) was treated with acetone, filtered, and pulverized. The pteridines were extracted by boiling the epidermis (100 g., wet) with 800 ml. of 50% ethanol for 20 min.; the solution was then filtered. The extraction with 50% ethanol was repeated twice, after which the yellow filtrates were combined and evaporated to dryness in vacuo. The residue was dissolved in water, and the solution was passed through an Ecteola cellulose column (7×12 cm.). The yellow fluorescent material (xanthopterin-B_2) was concentrated in a band at the top of the column; after the column had been throughly washed with water, this band was eluted with 0.01 N acetic acid. The eluate was concentrated, and the solution was again passed through an Ecteola cellulose column (3×10 cm.). The yellow eluate obtained by washing this with a 1% ammonium chloride solution was evaporated to a small bulk. The extract was then passed through a Filtrol (grade 58) column (2×7 cm.). After the column had been thoroughly washed with water, the band was eluted with 10% aqueous acetone. The eluate was concentrated to dryness in vacuo. All the above procedures were carried out in the dark in order to avoid photolysis. Four hundred grams of the epidermis gave 15 mg. of the crude product in a repetition of this procedure. From this about 2 mg. of the pure material was obtained by crystallization from methanol and water. The ultraviolet absorption spectra of the compound are shown in Fig. 1.

Air Oxidation in a Borax Solution.[5]—A small amount of the compound was dissolved in 5% aqueous borax and then shaken at 35°C for 24 hr. in the dark in order to avoid photodecomposition; the yellow color of the solution thereby gradually changed to a weak yellowish green, and the yellow fluorescence, to green. The product was purified by paper chromatography using as solvents *n*-propanol - 1% ammonia (2 : 1) and *n*-butanol - acetic acid - water (4 : 1 : 1). The absorption spectra, R_f-values and electrophoretic behavior

1) Y. Hirata, K. Nakanishi and H. Kikkawa, This Bulletin, **23**, 76 (1950).
2) S. Nawa and T. Taira, *Proc. Japan Acad.*, **30**, 632 (1954).
3) H. Aruga, S. Kawase and M. Akino, *Experientia*, **14**, 182 (1958).
4) M. Tsusue and M. Akino, *Zool. Magazine Japan*, **74**, 91 (1965).
5) S. Nawa, This Bulletin, **33**, 1555 (1960).
6) M. Goto, M. Kinishi and M. Tsusue, ibid., **38**, 503 (1965).

Table I. Paper chromatography and electrophoresis of xanthopterin-B_2 and its degradation products

Substance	Paper chromatography solvent					Electrophoresis*
	1	2	3	4	5	
Xanthopterin-B_2	0.24	0.54	0.42	0.50	0.43	0
Air oxidation product (in borax solution)	0.36	0.51	0.62	0.60	0.60	10
6-Hydroxylumazine	0.36	0.51	0.62	0.60	0.60	10
$KMnO_4$ oxidation product	0.15	0.28	0.32	0.35	0.58	48
Lumazine-6-carboxylic acid	0.15	0.28	0.32	0.35	0.58	48
$NaBH_4$ reduction product	0.31	0.45	0.45	0.55	0.59	0
Lumazine	0.31	0.45	0.45	0.55	0.59	0

Solvents: 1, *n*-butanol, acetic acid, water (4 : 1 : 1)
2, isopropanol, 1% ammonia (2 : 1)
3, *s*-butanol, formic acid, water (8 : 2 : 5)
4, *n*-propanol, 2% ammonium acetate (1 : 1)
5, 3% ammonium chloride

* Distance (in mm.) to anode after electrophoresis at pH 4.65 (sodium acetate buffer) for 1 hr. at 36 V./cm.

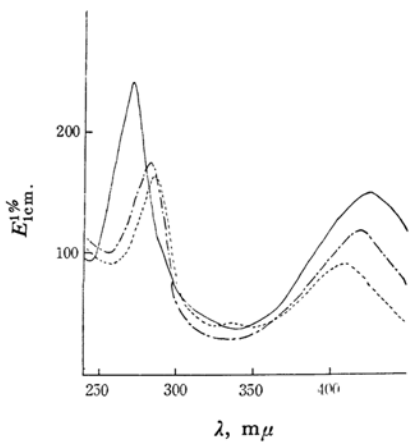


Fig. 1. The ultraviolet absorption spectra of xanthopterin-B_2: ——, at pH 12; —·—·, at pH 6.0; and ·····, at pH 1.0.

of the product were identical with those of an authentic sample of 6-hydroxylumazine[7] (Table I).

The Characterization of Lactic Acid.[8]—An aqueous solution of the compound (xanthopterin-B_2) (0.28 ml., c=41.0 μg./ml.) was treated with 5% borax (0.1 ml.) and shaken at 35°C for 24 hr. in the dark. The solution was then diluted with water to 1 ml. and heated at 100°C for 5 min. with 6 ml. of sulfuric acid and 0.05 ml. of 2% copper(II) sulfate. After cooling, 0.1 ml. of a 1.5% *p*-hydroxybiphenyl reagent was added; the mixture was allowed to stand at 30°C for 30 min. and then heated at 95°C for 1—2 min. The color which developed was estimated at 570 mμ (Hitachi spectrophotometer EPS-3). From a standard curve obtained with known amounts of lithium lactate, 41.0 μg. (1 ml. of the reaction mixture) was estimated to give 13.1 μg. of lactic acid (84% of the theoretical yield).

Potassium Permanganate Oxidation.—An aqueous solution of the sample (0.04 ml., c=5 mg./ml.) was treated with a saturated solution of potassium permanganate in 0.1 N sodium hydroxide (0.08 ml.) at 95°C for 30 min. The excess permanganate was then destroyed with alcohol, and the solution was filtered

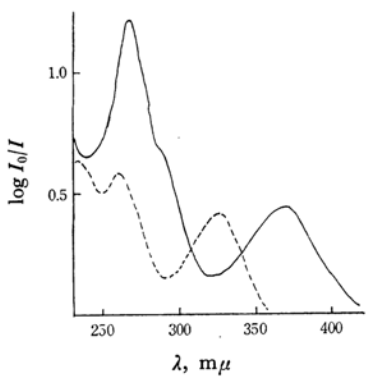


Fig. 2. The ultraviolet absorption spectra of the pteridine obtained by permanganate oxidation of xanthopterin-B_2: ——, at pH 12; and ·····, at pH 1.

in order to remove the manganese(IV) oxide. The product was identical with lumazine-6-carboxylic acid[9] (Table I and Fig. 2).

Periodate Oxidation.—A small amount (enough for paper chromatography) of an aqueous solution of the sample was treated with sodium metaperiodate at 30°C for 24 hr. and then submitted to paper chromatography and electrophoresis. The product was identical with lumazine-6-carboxylic acid.

Reduction with Sodium Borohydride.—An aqueous solution of the compound (xanthopterin-B_2) (0.1 mg./10 ml.) was treated with sodium borohydride (1 mg.); the greenish yellow fluorescence of the solution

7) A. Albert, J. H. Lister and C. Pedersen, *J. Chem. Soc.*, **1965**, 4621.
8) S. B. Barker and W. H. Summerson, *J. Biol. Chem.*, **138**, 535 (1941).
9) R. B. Angier, J. H. Boothe, J. H. Mowat, C. W. Waller and J. Semb. *J. Am. Chem. Soc.*, **74**, 408 (1952).

thereby almost disappeared. When the reaction mixture was shaken in air, its fluorescence became blue. The paper chromatography of this solution showed the presence of three fluorescent compounds. The major spot was lumazine-6-carboxylic acid and the intermediate lumazine; this was confirmed by the ultraviolet absorption spectra and the electrophoretic migration (Table I).

The Reaction of 2, 5-Diamino-4-hydroxy-6-methylaminopyrimidine with Glyoxal.—2-Amino-4-hydroxy-6-methylamino-5-nitropyrimidine was prepared from 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine and aqueous methylamine.[10] The product (6.0 g.) was purified by dissolution in 8% aqueous ammonia (100 ml.) (charcoal) and by reprecipitation with hydrochloric acid (6 N).

Found: C, 27.6; H, 3.3; N, 30.6. Calcd. for $C_5H_7N_5O_3 \cdot HCl$: C, 27.3; H, 3.6; N, 31.5%.

The pyrimidine hydrochloride (2 g.) in hot 3% ammonia (5 ml.) was treated with sodium dithionite. The solution was neutralized with acetic acid (pH 6), 8 ml. of glyoxal (40%) was added, and the whole was refluxed for 30 min. under nitrogen. The precipitate was collected by centrifugation and purified by chromatographic methods using a cellulose column (9×50 cm.; developer: isopropanol/1% ammonia=2 : 1) and a Dowex 1X8 column (5×24 cm.; $HCOO^-$; developer: 0.03 M formic acid/ammonia, the pH value being changed continuously from 9.4 to 7.0). The effluent containing the main fluorescent band was acidified with hydrochloric acid (pH 2—3), and the compound was adsorbed on Darco G-60, which was then washed well with water. The pteridine was eluted with 2% ammonium hydroxide-ethanol (3 : 1), the eluate evaporated to dryness in vacuo, and the residue crystallized from water to give a faint yellow product (20 mg.).

Found: C, 43.3; H, 2.3; N, 33.9. Calcd. for $C_6H_4N_4O_2$: C, 43.9; H, 2.5; N, 34.1%.

The product was indistinguishable from synthetic lumazine on the basis of ultraviolet, infrared and mass spectral data and R_f-values.

The Reaction of 2, 5-Diamino-4-hydroxy-6-methylaminopyrimidine with Monochloracetone.—2-Amino-4-hydroxy-6-methylamino-5-nitropyrimidine hydrochloride (2 g.) in hot 16% ammonia (50 ml.) was treated with sodium dithionite (8 g.), and then the solution was adjusted to pH 5 with acetic acid. After the addition of monochloracetone (6.0 ml.) it was refluxed for 1 hr. and concentrated to a small bulk in vacuo. By the use of a Dowex 1X8 column ($HCOO^-$), two main products (A and B) were separated from the reaction mixture.

Product A (24 mg.): colorless needles; m. p. 246—248°C (decomp.).

Found: C, 47.5; H, 3.3; N, 31.2. Calcd. for $C_7H_6N_4O_2$: C, 47.2; H, 3.4; N, 31.5%. λ_{max} mμ ($E^{1\%}_{1cm.}$) 248 (842), 360 (408) in 0.1 N sodium hydroxide and 327 (560) in 0.1 N hydrochloric acid.

Product B (14 mg.): faint yellow needles; m. p. 266—268°C (decomp.).

Found: N, 31.7. Calcd. for $C_7H_6N_4O_2$: N, 31.5%. λ_{max} mμ ($E^{1\%}_{1cm.}$) 256 (995), 368—372 (349) in 0.1 N sodium hydroxide and 232 (724), 333 (480) in 0.1 N hydrochloric acid.

Product A was indistinguishable from authentic 7-methyllumazine, while product B was identical with 6-methyllumazine, as determined by a study of the ultraviolet, infrared and mass spectral data and the R_f values. On oxidation compounds A and B gave lumazine-7-carboxylic acid and lumazine-6-carboxylic acid respectively.

Discussion

The new naturally-occurring compound described above gives ultraviolet spectra similar to those of sepiapterin. The presence of the parent lumazine ring system was demonstrated by reduction with $NaBH_4$ and by the reoxidation of the reduction product. This yielded lumazine. The production of lumazine-6-carboxylic acid by oxidation (potassium permanganate and sodium periodate) showed that there was a substituent on the 6-position, while its nature was demonstrated by the fact that the pteridine, on air oxidation in a borax solution, yielded 6-hydroxylumazine and lactic acid. From these findings, it may be concluded that the compound is a 6-lactyllumazine. No direct evidence for the presence of the reduced ring system was obtained; however, the similarity of the chemical properties and ultraviolet spectra (in basic, acidic and neutral media) to those of sepiapterin strongly suggests that it is 7, 8-dihydro-6-lactyllumazine. The 7, 8-dihydro structure of sepiapterin was proved by its nuclear magnetic resonance spectrum*.

In 1962 Kaufman[11] reported that sepiapterin functions as a cofactor in the enzymatic transformation of phenylalanine to tyrosine (the activity is 25 times that of folic acid). The activity of the compound (xanthopterin-B_2) as the cofactor was, however, only one-fourth that of sepiapterin.**

The question then arises as to the origin of this compound. A possible explanation is that it is produced enzymatically from reduced lumazine in exactly the same way that sepiapterin or isosepiapterin can be synthesized from reduced 2-amino-4-hydroxypteridine and an active C_3-compound.[12,13] Current theory supposes that the pteridine ring arises from guanosine or guanosine-5′-phosphate. The first step of the reaction involves the opening of the imidazole ring of the guanine moiety and the elimination of the carbon-8 of the purine.[14] The resulting 2, 5-diamino-4-hydroxy-6-D-ribofuranosylaminopyrimidine could then undergo transformation to lumazine in the presence of a two-carbon unit in just the same way

10) R. Lohrmann and H. S. Forrest, *J. Chm. Soc.*, **1964**, 460.

* Unpublished.

** M. Matsubara, personal communication.

11) S. Kaufman, *J. Biol. Chem.*, **237**, PC 2712 (1962).

12) S. Nawa and H. S. Forrest, *Nature*, **196**, 169 (1962).

13) F. I. Maclean, H. S. Forrest and J. Myers, *Biochem. Biophys. Res. Commun.*, **18**, 623 (1965).

14) F. Weygand, H. Simon, G. Dahms, M. Waldschmidt, H. J. Schliep and H. Wacker, *Angew. Chem.*, **73**, 402 (1961).

as lumazine can be synthesized from 2, 5-diamino-4-hydroxy-6-methylaminopyrimidine and glyoxal in vitro. Attempts to obtain 6-lactyl-7, 8-dihydrolumazine from reduced lumazine and α-keto-β-hydroxybutyric acid in the presence of thiamine, were, however, fruitless. An alternative pathway, in which xanthopterin-B_2 is derived enzymatically from xanthopterin-B_1, is more probable. The presence of sepiapterin-deaminase in *B. mori lem* was actually demonstrated by one of us (M. T.). This finding will be reported on elsewhere.

In a preliminary communication,[6] we reported that the oxidation product of the compound (xanthopterin-B_2) (potassium permanganate and potassium poriodate) is indistinguishable from the synthetic "2-amino-4-hydroxy-8-methyl 4, 8-dihydropteridine-6-carboxylic acid" obtained by the condensation of 2, 5-diamino-4-hydroxy-6-methylamino-pyrimidine with β-bromopyruvic acid, and that a reduction product ($NaBH_4$) is identical with the "2-amino-4-oxo-8-methyl 4, 8-dihydropteridine" prepared by the condensation of the pyrimidine with glyoxal.[15] From these observations and its behavior upon air-oxidation in a borax solution, the N_8-methylsepiapterin structure was tentatively postulated. However, the present investigation has proved that the condensation of 2, 5-diamino-4-hydroxy-6-methylaminopyrimidine with β-bromopyruvic acid or glyoxal under the conditions described here does not give the desiredp roducts, but, that unexpectedly, lumazine-6-carboxylic acid or lumazine is formed. In the same way, the condensation of the pyrimidine with monochloracetone yields 6-methyllumazine and its 7-isomer. Previously, we were not aware of this unusual reaction; this led to an incorrect formulation of xanthopterin-B_2. We now wish to present the structure II for this compound.

15) D. J. Brown and N. W. Jacobsen, *J. Chem. Soc.*, **1961**, 4413.

Summary

On the basis of degradative experiments, the structure of 7, 8-dihydro-6-lactyllumazine has been postulated for a yellow pigment from *Bombyx mori lem.*

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