New Pterins (Xanthopterin-B, Leucopterin-B and a 6-Dehydroxyleucopterin[1] Derivative) Obtained from *Bombyx mori* (Silk Worm)

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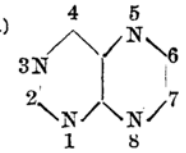
According to Kikkawa[2] and others, the various pigments of *Bombyx mori* (riborflavin of the Malpighian tubes, urochrome, the eye-, epidermis-, egg- and red-excrement pigments) are derived from tryptophan through the common precursor, +chromogen. +Chromogen corresponds to the so-called cn$^+$ substance[3][4]

(1)



(According to the *Ring Index*)

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(2) H. Kikkawa, *Kagaku*, **19**, 414 (1949).

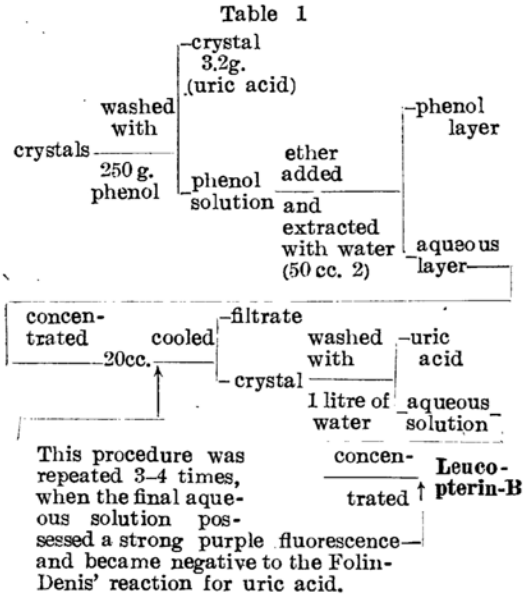
(3) G. W. Beadle *Genetics*, **22**, 587 (1937).

(4) B. Ephrussi, *Am. Nat.*, **72**, 5 (1938).

of *Drosophila melanogaster* or hormone A of *Ephestia kühniella*[5][6] and the resolution of its chemical nature is of great importance. In order to clarify the nature[7] of this substance in the case of silk-worms, as well as to establish this hypothesis, we are undertaking chemical researches of the various pigments and + chromogen. The present paper deals with the epidermis pigments of normal and mutant types.

Leucopterin-B.—400 larvae (normal type, China 108) in the fifth stage were dissected, the epidermis dried, powdered, treated four hours with ether in a modified Soxhlet apparatus, denatured with ethyl alcohol and the tissues extracted with 2400 cc. of water. This was concentrated to 140 cc. and made acidic to 0.1 *N* with hydrochloric acid. The crystals obtained by cooling this solution overnight in an ice-box, were submitted to the following procedure (Table 1).

Table 1



Leucopterin-B was likewise obtained from the mutants, "od" ("d-oily")[8] and "lem" ("lemon-colored")[9]. A solution of leucopterin-B at *p*H 7 possessed a strong purple fluorescence. It was soluble in water, alkali and phenol; and insoluble in alcohol, ether and dilute hydrochloric acid. The ultra-violet absorption spectrum is showh in Fig. 1. The

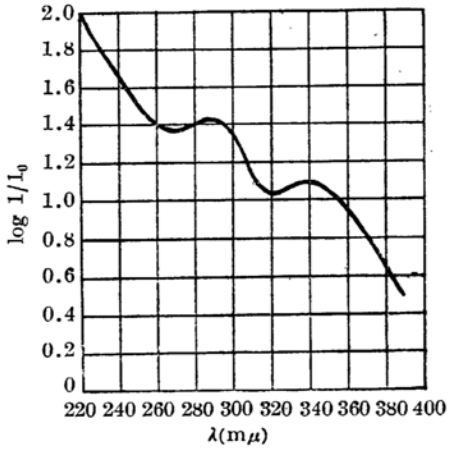


Fig. 1.—Ultraviolet absorption spectrum of leucopterin-B (in 0.1 *N* NaOH). Since the substance was unobtainable in a crystalline state, the concentration of the solution was obscure and thus the extinction coefficient could not be calculated.

maximum located at 290 mμ and 340 mμ in 0.1 *N* sodium hydroxide, is not identical with the other known pterins. It begins to decompose from 250°. These natures suggested it to be a pterin. The name leucopterin-B was proposed; the suffix B derived from **Bombyx**. The Folin-Denis' reaction was negative.

Uric Acid.—(See Table 1). Though the crystals were recrystallized seven times by dissolving in 0.1 *N* sodium hydroxide and acidifying, a fluorescent substance (leucopterin-B) was still extracted by boiling in water. Nevertheless, elementary analysis (C, H, N), ultraviolet absroption spectra, qualitative reactions and chemical behavior showed it to be uric acid. As pointed out by Jucci[10] uric acid seemed to exist as salt in the epidermis. When cooling the original boiling water extract, precipitates appeared, which when ignited gave a residue which was identified as being calcium carbonate. This suggests that uric acid might probably exist as a calcium salt.

Xanthopterin-B.—It was reported in our previous paper[11] that the yellow pigment from the mutant "lem" was xanthopterin, notwithstanding the existence of some obscure points which remained to be clarified (such facts as the photolysis of the pigment). But further experiments have obliged us to revise this conception and the new xanthopterin-like substance has been named xanthopterin-B, the

(5) E. Caspari, *Arch. Entw.-mech.*, **130**, 353(1933).
(6) A, Kühn, E. Caspari and E. Plagge, *Z. Nachr. ges. Wiss.*, **2**, 1 (1935).
(7) According to a private communication to Kikkawa from Dr. Beadle the structure of the substance (obtained from *Calliphora*) corresponding to +chromogen is reported to be 3-hydroxykynurenine. Furthermore, we (Hirata, Nakanishi and Kikkawa) have succeeded in isolating + chromogen from *Bombyx mori* in a crystalline state.
(8) "od": d-oily or distinct translucent, situated on the locus 49.6 of the first chromosome.
(9) "lem": lemon-colored, situated on the locus 0.0 of the third chromosome.
(10) C. Jucci, *Proc. 6th. International Congress of Genetics*, Vol. I, (1932).
(11) Y. Hirata, K. Nakanishi and H. Kikkawa, *Science*, in press.

suffix B coming from Bombyx. The extraction was carried out in the dark to avoid photolysis. The ether-treated and denatured tissues, after being extracted with 350 cc. of 2 *N* hydrochloric acid, were submitted to the procedure previously stated when uric acid and leucopterin-B were obtained. The hydrochloric acid solution was extracted from the filtrate with 100 cc. phenol and re-extracted with 100 cc. water by means of the addition of ether. The aqueous solution was evaporated when a minute amount of yellow powder (xanthopterin-B) resulted. Xanthopterin-B was soluble in water, acid, alkali and phenol, and insoluble in ether. The ultraviolet absorption spectrum in 0.1 *N* sodium hydroxide (Fig. 2) has a band at 265 mμ and a shoulder at about 350 mμ, which is not identical with xanthopterin. Photolysis is described in the previous paper[11]. The Folin-Denis' reaction was slightly positive. It is to be noted that both leucopterin-B and xanthopterin-B are reduced reversibly by sodium hydrosulfite into a non-fluorescent leuco-derivative, which suggested the possibilities of these pterins to play a vitamin B_1 and B_2-like role in the oxidation-reduction system of living cells, similar to those observed in the case of xanthopterin[12] and fluorescyanin[13] (or ichtyopterin).

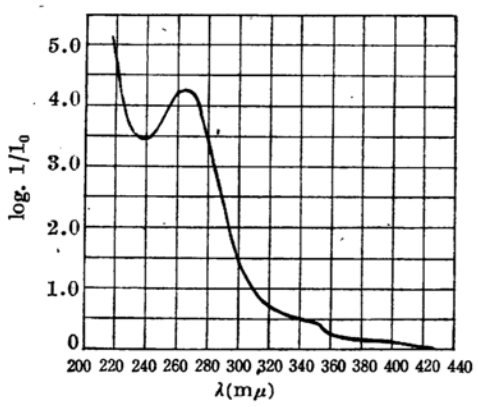


Fig. 2.—Ulraviolet absorption spectrum of xanthopterin-B (in 0.1 *N* NaOH). Since the substance was unobtainable in a crystalline state, the concentration of the solution was obscure and thus the extinction coefficient could not be calculated.

Paper Chromatography of the Pterins.—(Table 2). The method described by Good and Johnson[14] was employed, using the butyl alcohol-acetic acid mixture of Partridge,[15] and characteristic fluorescence for location of the individual pterins. The following general method was employed for preparing the samples: the denatured tissues were extracted with boiling water, ammonium sulfate added, the

Table 2

One-dimensional Paper Chromatography of Various Extracts and Pterins

Name of substance	Leucopt.	Decompd. product of xantho. B	Decompd. product of xantho.	Leuco. B	Xantho.	Xantho. B
Color of fluorescence	pale-blue	bright-blue	blue	purple	sky-blue	yellowgreen
R_f value	0.12	0.26	0.30	0.33	0.41	0.50
Silk worm						
normal	+			+		
"lem"	+			+		+
"lem" (photolysis)	+	+		+		
"odd"	+			+		
Butterfly						
Pieris rapae crucivora B	+			+		
Eurema laeta B	+			+	+	+
Eurema laeta B (photolysis)	+	+		+	+	
Xanthopterin			+		+	
Ichtyopterin or fluorescyanin (*Cyprinus carpio L*)				+*		
Pyrrol-chrome (*Rana nigromaculata*)		0.24 (light blue)		+*	0.40 (blue)	0.47 (blue)

* The R_f values merely coincided with leucopterin-B but the substances were not identical.

(12) W. Koschara and H. Haug, *Z. physiol. Chem.* **259**, 97 (1939).
(13) R. G. Busnel, F. Chauchard, H. Mazone, M. Pesson and M. Polonovski, *Compt. rend. Soc. Biol.*, **137**, 594 (1943).
(14) P. M. Good and A. W. Johnson, *Nature*, **163**, 31 (1949).
(15) Partridge, *Biochem. J.*, **42**, 238 (1943).

precipitate discarded, pigment extracted into liquid phenol and returned to water by the addition of ether. In case of silk-worms, the separated pure pigments were employed as well. The scales of a carp (*Cyprinus carpio L*) were used as an ichtyopterin[16] (or fluorescyanin[17]) source. Xanthopterin was received from Dr. E. L. Rickes, Merck and Co., Inc., and pyrrol-chrome[18] from Dr. T. Goda, the University of Tokyo, to which we are greatly thankfull.

Though ichtyopterin (fluorescyanin) and leucopterin-B possessed the same R_f values (0.33), the two differed with respect to ultraviolet absorption spectrum and the change of fluorescence accompanying the change in *p*H (mentioned below). The purple fluorescent pigment found in Pierids seemed to be identical with ichtyopterin (from R_f value and fluorescence behavior).

Pyrrol-chrome[18], a fluorescent and reducing material obtained from the dorsal skin of the frog, *Rana nigromaculata*, gave a similar spot besides three other spots. The chemical nature of pyrrol-chrome is obscure, but this fact indicated the possibility of some relationship with the other pterins.

As stated by Becker and Schöpf[19], and Good and Johnson,[14] the nature of the blue spot at 0.30 is obscure. When a 0.1 *N* sodium hydroxide solution was dropped on this spot, the fluorescence changed into a greenish tone, whilst that of the spots at 0.26 and 0.33 faded out under similar treatments: this indicated in a simple manner, the unidentity of these spots.

Fluorescence. (a) **Leucopterin-B.**—The fluorescence was the strongest at *p*H 4-8 (Table 3). mediums and in acetic acid.

Table 3

Fluorescence of Leucopterin-B and Ichtyopterin

	*p*H 1.6 (HCl)	*p*H 2.8 (AcOH)	*p*H 7.0	*p*H 9.6 (NH_4OH)	*p*H 12.6 (NaOH)
Leucopterin-B	feeble	purple	purple-blue	blue	purple-blue
Ichtyopterin*	feeble	purple	purple-blue	pale-blue	pale-blue

* The purple fluorescent substance in the extracts of *Pierids* also behaved similar to ichtyopterin.

The change of fluorescence observed for the purple fluorescent materical in pyrrol-chrome was rather akin to ichtyopterin than to leucopterin-B.

(b) **Xanthopterin-B.**—It behaved similar to xanthopterin, in acidic, alkaline and neutral

With the mutant "od", the fluorescence of the extracts as well as the spot on the paper chromatogram were feeble, and the amount of uric acid also very small. These explain the transparency of the skin of this mutant.

A Crystalline 6-Dehydroxy-leucopterin[1] Derivative.—We had unfortunately missed to record the exact species of this characteristic silk-worm, which at first seemed to belong to the normal white types, and were thus unable to obtain this material for the second time, in spite of many repeated extraction procedures. Thus its presence suggested the possible existence of white mutants which are passed over due to their similar external aspects.

The boiling water extract of 50 larvae was acidified and cooled when crystals (uric acid) and white powder were obtained. The white powder was boiled with 2*N* hydrochloric acid, dissolved and the solution cooled, upon which a considerable amount of colorless plates precipitated. This was recrystallized from the same solvent and the following natures were observed. The solid crystal (decomposition point: over 270°) had a purple fluorescence, and formed yellow sodium and ammonium salts, the solution possessing a green or light bluish fluorescence. The yellow alkali solution (0.1%) faded out into a colorless solution when left for a day. It gave a positive murexide test, and Folin-Denis' reaction, and dissolved in fuming hydroiodic acid with the precipitation of iodine.

On dilution of the solution, the iodine was reduced and the pterin reprecipitated. These natures were identical with those described for isoxanthopterin but the ultra-violet absorption spectrum (Fig. 3) was not identical, the max-

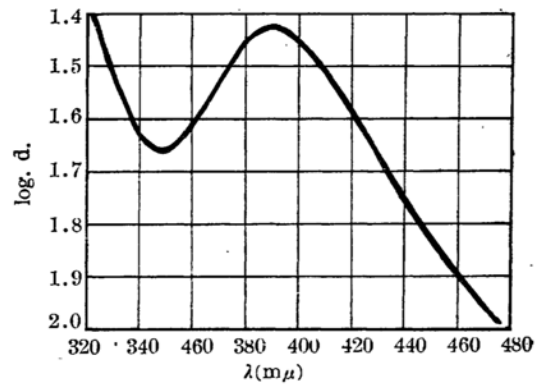


Fig. 3.—Ultraviolet absorption spectrum of the 6-dehydroxy-leucopterin derivative (in 0.1 *N* NaOH). d: lenght of tube concentration: 0.1%

imum being located at 390 mμ, as compared with the values 252 mμ and 340 mμ for isoxanthopterin[16]. But the behavior with hydroiodic acid indicated this pterin to be a derivative of 6-dehydroxy-leucopterin.[1] The sus-

(16) R. Hüttel and G. Sprengling, *Ann.* **554**, 69 (1943).

(17) M. Polonovski, R. G. Busnel and M. Pesson, *Compt. rend. Acad. Sci.*, **217**, 163 (1943).

(18) T. Goda, *J. Fac. Sci., Imp. Univ. Tokyo*, Section IV, **5**, 305 (1941).

(19) Becker and Schöpf, *Ann.*, **524**, 49, 124(1936).

pension in acetic anhydride was clarified by the addition of sulfuric acid, which demonstrated the presence of a hydroxyl group.

Summary.—Chemical researches on the epidermis pigments of a normal type (China 108) and mutants ("lem" and "od") of *Bombyx mori* have been carried out in order to elucidate the tryptophan metabolism. Besides uric acid as its calcium salt (which was commonly distributed), the following three new pterins were obtained from the respective races:

(a) Normal type: leucopterin-B, leucopterin.
(b) Mutant "lem": leucopterin-B, xanthopterin-B and leucopterin.
(c) Mutant "od": leucopterin-B, leucopterin.
(d) Unidentified white race: 6-dehydroxy-leucopterin derivative.

Leucopterin-B and ichtyopterin, xanthopterin-B and xanthopterin, the 6-dehydroxy-leucopterin derivative and isoxanthopterin, respectively, showed many superficial similarities but differed in their ultraviolet absorption spectrum and several other natures. The pterin nature of the epidermis pigments being established, we have acquired a new information related to tryptophan metabolism and +chromogen.

In conclusion the authors wish to acknowledge their indebtedness to Prof. F. Egami for his advices and encouragements given in the course of this work and Prof. K. Yamasaki and Mr. K. Sone for the measurement of the ultraviolet absorption spectrum. We also gratefully acknowledge the precious gifts from Drs. E.L. Rickes and T. Goda. The advices given by Dr. T. Hama, the University of Tokyo, have also benefited us a great deal. The cost of this research has been defrayed from the Scientific Research Encouragement Grant from the Ministry of Education, to which the authors' thanks are due.

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