Crystalline + Chromogen Obtained from *Bombyx mori*

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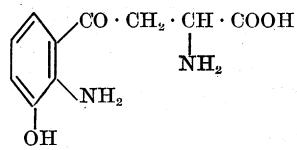
Corresponding to the cn$^+$-substance of *Drosophila* and A-substance of *Ephestia*, + chromogen (W-substance of *Bombyx*) is widely distributed among insects and other arthropods. Most of its chemical nature, including its structure, has been obscure, except that one of us (*3*) in 1943 isolated a minute amount of a crystal-like substance which seemed to be + chromogen. Recently W. Weidel (*1, 2*), of the Kaiser Wilhelm Institut für Biochemie, has isolated the cn$^+$-substance from larvae of *Calliphora erythrocephala* and stated it to be 3-hydroxykynurenine, which was postulated by Mitchell and Nyc (*4*) to be a precursor of nicotinic acid, but the details are unknown.

The authors have isolated 50–60 mg of light-yellowish crystals from 400 g of new-laid eggs of *Bombyx mori*. The qualitative chemical nature of this crystalline substance, as well as results of biological tests, provides convincing evidence for considering it to be + chromogen. Elementary analysis and certain aspects of chemical behavior coincide with the structure shown in Fig. 1, but



(I)

FIG. 1.

direct proof is not yet available. The synthesis of 3-hydroxykynurenine, which is at present in progress, will determine the validity of this postulated structure. The isolation was carried out by modifying the method of Kikkawa (*3*).

Within 24 hr after oviposition 400 g of eggs of the normal type were killed in methanol, the methanol discarded, and the eggs carefully ground. This was extracted at 75° C with methanol until the extract and residue showed a negative Ehrlich's reaction. Methanol was thoroughly removed by concentrating the combined extracts *in vacuo*, and the remaining aqueous solution was washed with ether and the ether then removed. Hopkins-Cole's reagent was added, and the mixture was left in an icebox for 2 days, when precipitates appeared. These were suspended in 2% H_2SO_4; H_2S was passed through the suspension, and the resulting HgS discarded. The filtrate was concentrated to a H_2SO_4 concentration of 5% and 2–3 vol of 20% phosphotungstic acid in 5% H_2SO_4 were added. The solution was ice-cooled one night, whereupon precipitates of + chromogen separated. After being washed with the phosphotungstic acid solution, the precipitate was added to 5% H_2SO_4, and the phosphotungstic acid solution removed by shaking with an ether-amyl alcohol mixture (125: 100). A small amount of precipitate was filtered, and $BaCO_3$ added till the solution became red against Congo red. The $BaSO_4$ precipitate was washed thoroughly until the washing liquid was negative to Ehrlich's reagent. The filtrate was added to the original solution, and the combined solutions concentrated *in vacuo*, when a yellow-brownish solid was obtained. This was extracted several times with methanol; + chromogen dissolved, leaving an insoluble brownish mass. The extracts were concentrated *in vacuo* and left in an icebox; crystalline + chromogen separated. If necessary, the methanol-extraction was repeated. The crystals could be recrystallized from a small amount of water. Yield: 50–50 mg; 3–4 mg of the substance was also obtained from eggs of the white-2 mutant of *Bombyx mori*. The crystals, light-yellowish needles, mp 180° C (accompanying decomposition), possessed approximately 1 mole of water of crystallization, which was removed by heating to 100° C *in vacuo* for 2 hr.

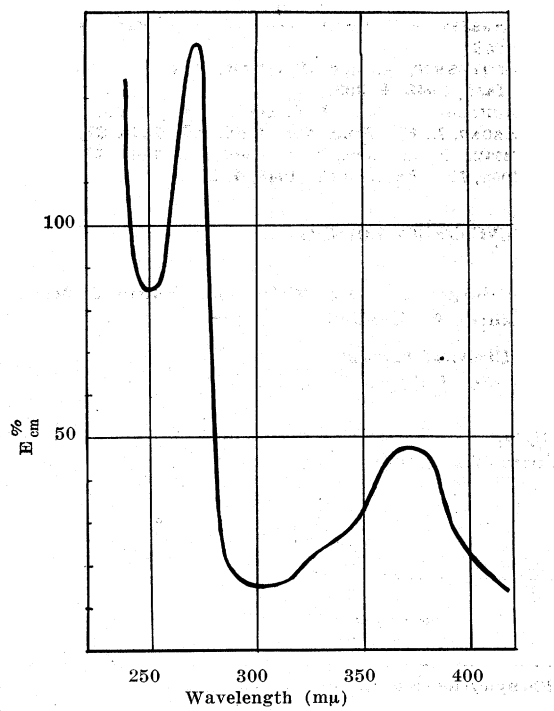


FIG. 2. Absorption spectrum of + chromogen in water.
Abs. maximum : 270 mμ ($E^{\%}_{cm} = 143$).
370 mμ ($E^{\%}_{cm} = 48$).

Anal. calculated for structure I ($C_{10}H_{12}O_4N_2$):

C 53.59, H 5.40, N 12.49.

Found: C 52.23, H 6.04, N 12.80.

Assuming that the substance obtained is actually 3-hydroxykynurenine, the analytical data suggest that a further amount of water of crystallization may still re-

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main. The ultraviolet absorption spectrum (Fig. 2) closely resembled that of kynurenine, but it gave a negative Otani's kynurenine reaction (*5*). The ninhydrine, xanthoprotein, and Ehrlich's diazo reactions were positive. The alkaline solution was of a deep-yellowish color and gave a jasminelike odor when heated. Dilute $KMnO_4$ solution also changed the color to deep yellow (urochromogen test of Weiss). The Folin-Denis' uric acid reagent changed the solution to a greenish, and then to a bluish, color. The murexide and lumiflavin tests were negative.

Biological tests employing the mutants vermilion and cinnabar of *Drosophila* were positive.

Addendum: In a previous paper in this journal (*Science*, 1950, **111**, 608) concerning the tryptophan pigments, which dealt with the epidermis pigments of the yellow mutant "lem" of *Bombyx mori*, we reported that the yellow pigment was xanthopterin, but, according to direct comparison with a sample of xanthopterin (kindly sent by E. L. Rickes, Merck & Co., Inc.) it has been found that, although this pigment resembles xanthopterin remarkably closely, it is slightly different. It has been named xanthopterin-B (B from *Bombyx*), and 2 other new pterins have also been isolated (leucopterin-B and a 6-dehydroxyleucopterin derivative) (*6*).

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Built-up Poultry-House Litter as a Growth-promoting Supplement for Chicks on an All-Vegetable Vitamin B_{12}-deficient Diet

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Extensive trials by Kennard and Chamberlin (*1*, *2*) at the Ohio Agricultural Experiment Station demonstrated that poultry-house floor litter which has accumulated (built up) for a long period of time gives better chick growth on all-vegetable diets than litter which is completely changed at frequent intervals. Reports have also been made regarding improved hatchability of eggs from hens on such litter (*3*, *8*). It has been found that poultry droppings contain the "animal protein factor" (APF) (*7*), and that incubation of hen feces for 72 hr at 30° C stimulates further synthesis of this factor (*4*).

In a series of studies dealing with the synthesis of vitamin B_{12} (now recognized as being an important part of the animal protein factor) by microorganisms in poultry-house litter, marked stimulation of growth was secured by adding built-up litter to an all-vegetable vitamin B_{12}-deficient diet fed to S. C. White Leghorn chicks in a wire-floored battery brooder.

The basal diet used was one consisting principally of soybean oil meal and ground yellow corn supplemented with the known requirements for vitamins, minerals, and the amino acid methionine. The diet was used for studies of soybean oil meal and animal protein factor by workers at the Purdue Agricultural Experiment Station (*5*, *6*).

Vitamin B_{12} was shown to be the principal deficiency in the basal diet by the marked stimulation of growth secured by intramuscular injection of a saline solution of crystalline vitamin B_{12} (Cobione)[2] given in the amount of 0.3 μ/g per chick weekly. This injection was as effective in stimulating chick growth as feeding the crude APF supplement[2] containing the equivalent of 20 μ/g of vitamin B_{12}/kg of diet.

As shown in Table 1, addition of 1%, 2.5%, and 5% levels of screened unautoclaved built-up corncob litter (over 1 year old) to the basal diet gave increased chick growth to 6 weeks of 74, 45, and 67 g, respectively, as compared to 84 g for the added APF supplement fed at the rate of 20 μ/g of vitamin B_{12}/kg of diet. The built-up litter used for chick feeding was accumulated from hens that had been fed an all-vegetable vitamin

TABLE 1

EFFECT ON CHICK GROWTH OF ADDING BUILT-UP POULTRY-HOUSE LITTER AND OTHER SUPPLEMENTS TO AN ALL-VEGETABLE VITAMIN B_{12}-DEFICIENT DIET

Diet	No. Trials	Total No. chicks at start	No. died	Avg 6-wk wt in g (all trials)	Feed per g gain
Basal vitamin B_{12}-deficient diet	3	53	8	345	3.78
Basal + APF supplement*	3	53	4	429	3.23
Basal + vitamin B_{12} injections†	1	15	1	426	3.02
Basal + 2.5% ground corncobs	1	15	1	250	5.16
Basal + 5% ground corncobs	1	15	1	265	4.85
Basal + 1% unautoclaved built-up litter	1	18	0	419	3.04
Basal + 1% autoclaved built-up litter	1	18	1	394	3.28
Basal + 2.5% unautoclaved built-up litter	3	53	3	390	3.29
Basal + 2.5% autoclaved built-up litter	2	38	0	466	2.82
Basal + 5% unautoclaved built-up litter	2	27	3	412	3.34
Basal + 5% autoclaved built-up litter	2	35	0	493	2.55

* Twenty μ/g vitamin B_{12}/kg of diet.
† Crystalline vitamin B_{12} (0.3 μ/g/chick weekly).

built-up litter used for chick feeding was accumulated from hens that had been fed an all-vegetable vitamin

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[2] We are indebted to Merck & Co., Inc., Rahway, N. J., through the courtesy of D. F. Green, for the crystalline vitamin B_{12} (Cobione) and the APF supplement (crude vitamin B_{12}) used in these studies.