DEVELOPMENT OF EYE-COLORS IN DROSOPHILA:[1] BACTERIAL SYNTHESIS OF v+ HORMONE

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The development of eye-color in certain insects is controlled by specific diffusible substances[2] so far found only in insects. In Drosophila two substances, v^+ hormone and cn^+ hormone, are essential for pigment formation, and are not produced by flies homozygous for the genes *v* and *cn*, respectively. It is also known[3,4] that *v bw* animals grown at low levels of nutrition become capable of producing v^+ hormone and therefore develop pigmented eyes. This so-called "starvation effect" is prevented by the addition of sugar to the diet.

Khouvine, *et al.*,[3] reported that *v bw* larvae raised on peptone-glucose media containing tryptophane developed eye pigment while those grown on tryptophane-deficient media did not. These experiments, however, were not carried out under aseptic culture conditions. Since studies of nutrition in our laboratory have shown that such media will not support growth of Drosophila larvae under aseptic conditions, it seemed probable that growth of microörganisms made Khouvine's media adequate. These microörganisms may also have been concerned in the expression of the tryptophane effect, possibly through starvation.

TABLE 1

EFFECT OF TRYPTOPHANE AND GROWING *Bacillus sp.* ON EYE-COLOR OF *v bw D. melanogaster*

Figures in Parentheses Give Number of Adult Flies. (1.5 Per Cent Agar Medium 10 Cc. in 35 Cc. Vials.)

ADDITIONS TO MEDIUM	CULTURAL CONDITIONS	PROLONGATION OF LARVAL LIFE IN DAYS	EYE-COLOR*
			2.5–3.0
	Sterile	3–5	(9)
Dry yeast, 0.5 per cent	Inoculated with *Bacillus sp.*	4	2.0–3.0 (6)
Dry yeast, 0.5 per cent and	Sterile	2–5	0.0–1.0 (27)
sucrose, 2.0 per cent	Inoculated	1–2	0.5–2.0 (17)
Dry yeast, 0.5 per cent, sucrose, 2.0 per cent and tryptophane, 10 mg. per 10 cc.	Sterile	1–6	0.0–1.0 (40)
	Inoculated	1–2	2.0–5.0 (39)

* For significance of eye-color values cf. Tatum and Beadle.[6]

Preliminary experiments designed to test the effect of tryptophane on eye-pigment development in *v bw* Drosophila under aseptic conditions, as described elsewhere,[5] gave entirely negative results. One culture, however, was accidentally contaminated with an aerobic bacillus, and a marked effect on eye-color of the flies developing in it was observed. This unidentified *Bacillus sp.* was obtained in pure culture, and the effect of its growth on the development of eye-color was then investigated more thoroughly. The results given in table 1 show that neither the bacillus nor tryptophane separately had any significant influence on pigment production. However, in the presence of both the organism and tryptophane, eye pigmentation was greatly intensified. This shows conclusively that tryptophane is able to modify eye-color only through the intermediation of microörganisms.

These experiments did not eliminate a possible action of the organisms and tryptophane through starvation of the larvae, although the sugar concentration used should inhibit the starvation effect.[5] Accordingly, another experiment was made, using a medium containing 1.5 per cent agar, 0.5 per cent yeast, 3 per cent sucrose and 10 mg. tryptophane per 10 cc. Separate cultures were inoculated with the bacilli on the first, second and third days after hatching of the larvae. Even those flies developing on the medium inoculated on the third day developed eye-pigment (34 flies, eye-color 3.5 to 5.0[6]), although calculation of the total time to eclosion indicated that the bacteria had been introduced after most of the larvae had passed the 72-hour "critical period." After this time larvae are not modified by starvation.[4] It therefore seemed probable that the observed effect on eye-color was not caused by starvation. A more nearly direct test was made by transferring 72-hour-old, sterile, fully fed *v bw* larvae[5] to a 3-day-old culture of the *Bacillus sp.* growing on dry yeast sucrose-agar with tryptophane. Larvae over 72 hours old cannot be influenced by starvation. Nevertheless, the adult flies from this experiment showed definitely modified eye-color (22 flies, eye-color 1.0 to 3.5). In another similar experiment, the *Bacillus sp.* was grown on yeast sucrose-agar with tryptophane and the culture was autoclaved after 3 days. Fully fed, 72-hour-old, sterile *v bw* larvae were then placed aseptically on this autoclaved medium. The flies eclosed in normal time (215 hours from egg-laying) and were strongly modified, 36 flies, eye-color 3.0 to 3.5. Corresponding tests showed that *su*^{2}*-v, v;bw* flies were also strongly modified; 10 ♂♂, eye-color 3.0 to 3.5; 13 ♀♀, eye-color 3.5 to 4.0. Normal eye-color values of this stock, ♂♂ = 1.0, ♀♀ = 2.0[6]. On the other hand, *cn bw* flies were not affected at all (15 flies, eye-color 0.0).

The eye-color modification observed in these experiments was definitely not associated with a starvation effect. The concentration of sucrose used in all experiments would have inhibited almost completely any effect of

starvation.[5] Moreover, pigmentation was also produced in larvae which were too old to be affected by starvation.[4]

It has been found[7] that starvation of *v bw* larvae modifies the fat body so that after transplantation it will induce pigmentation of the eyes of a normal *v bw* host. Fat bodies from *v bw* larvae cultured on autoclaved tryptophane medium on which the bacterium had been grown were transplanted into normal *v bw* larvae. The results showed that the fat bodies were not modified as they would have been by starvation (12 flies, eye-color 0.0).

The evidence indicates conclusively that the eye-color change is due to the ingestion by the larvae of a specific substance, with v^+ activity, which is produced by the *Bacillus sp.* only in the presence of tryptophane. The v^+ and cn^+ hormones are known to be effective when taken in through the digestive system.[8] Extracts have been prepared from the autoclaved bacterial culture and injected into *v bw* larvae. Although these extracts were quite toxic, 20 pupae developed far enough to show eye-color (1.0 to 2.5). Six eclosed flies showed eye-color ranging from 0.5 to 2.7. The modification of *v bw* flies following injection of extracts is the most specific and critical available test for v^+ eye-color hormone. The method of extraction from the agar medium showed that the substance produced by the bacteria was soluble in water and ethyl alcohol, but insoluble in acetone and chloroform and was heat-stable. It therefore behaved in the same way as does v^+ hormone obtained from Drosophila pupae.[6] Another characteristic of v^+ hormone is that it is transformed into cn^+ hormone by *v bw* flies. A preliminary test showed that the bacterial product, which has only v^+ hormone activity, was also changed into cn^+ hormone in this way. Fully fed, 72-hour-old *v bw* larvae were fed on the autoclaved bacterial culture. Forty-eight hours after pupation, 15 of these were boiled, crushed and fed[8] under aseptic conditions to five *cn bw* larvae. Four flies developed and showed a definite eye-color modification of 0.3.

It should be pointed out that the active bacterial product resembles v^+ hormone in yet another way. All active v^+ (and cn^+) hormone fractions as yet prepared from Drosophila pupae[6] have been decidedly yellow in color. The active preparations from the bacteria show the same clear yellow color. It seems probable that this color is associated with the hormone itself, since the bacteria grown in the absence of tryptophane do not produce the active substance, and the yellow color is not developed.

A number of experiments have been carried out to determine the optimal conditions for the production of the effective substance by the unidentified bacillus. The organisms were grown 3 days on each medium. After autoclaving and slanting the medium, 72-hour-old, fully fed *v bw* larvae were introduced. The results (table 2) show that the active substance is produced only under aerobic conditions (solid medium or aerated liquid

TABLE 2

PRODUCTION OF SUBSTANCES WITH v^+ ACTIVITY BY *Bacillus sp.* IN 3 DAYS OF GROWTH UNDER VARIOUS CONDITIONS

Medium: dry yeast, 1.0 per cent; sucrose, 3.0 per cent; agar, 1.5 per cent; and tryptophane, 10 mg.; total volume, 10 cc. in 35 cc. vials. Figures in body of table represent eye-color values, those in parentheses give number of adult flies.

COMPLETE CULTURE MEDIUM	BACTERIAL CELLS FROM CULTURE MEDIUM*	CULTURE MEDIUM WITH BACTERIAL CELLS REMOVED	LIQUID CULTURE MEDIUM,** NON-AERATED	LIQUID CULTURE MEDIUM,** AERATED
3.0–3.5	0.5	3.0–3.5	0.0	3.0–3.5
(25)	(15)	(17)	(19)	(3)

* Cells added to fresh medium, autoclaved and tested.

** 1.5 per cent agar added to medium just before autoclaving.

medium), and that it readily diffuses from the bacterial cells into the medium.

The influence of the tryptophane concentration was then investigated, using solid medium and growing the bacilli 3 days before autoclaving the

TABLE 3

INFLUENCE OF TRYPTOPHANE CONCENTRATION ON PRODUCTION OF SUBSTANCE WITH v^+ ACTIVITY BY *Bacillus sp.*

(Basic agar medium and significance of figures as in table 2)

TRYPTOPHANE CONCENTRATION IN MG. PER 10 CC.						
0	1	5	10	20	40	70
0.0	0.1–0.5	2.0–3.0	3.0–3.5	3.0–3.2	2.5–3.0	2.0–3.5
(25)	(30)	(22)	(27)	(25)	(19)	(21)

culture and then adding 72-hour-old *v bw* larvae. The results (table 3) show that the optimal concentration of tryptophane under these conditions was 10 mg. per 10 cc. of medium. Higher concentrations did not increase the intensity of eye-pigmentation. Presumably, the bacteria can produce only a certain amount of the effective substance in 3 days, even when provided with an excess supply of tryptophane.

The results of a series in which the bacteria were allowed to grow on a medium containing 10 mg. tryptophane per 10 cc. for different periods of time before autoclaving, showed that the eye-color intensity increased with the time of bacterial growth (table 4). All the flies developing on the me-

TABLE 4

INFLUENCE OF TIME OF GROWTH OF *Bacillus sp.* ON THE PRODUCTION OF SUBSTANCE WITH v^+ ACTIVITY

(Medium and significance of figures as in table 2)

TIME OF BACTERIAL GROWTH IN DAYS				
1	3	5	7	10
1.0–2.0	3.0–3.5	4.0–4.5	4.5–5.0	5.0
(15)	(25)	(22)	(16)	(23)

dium cultured for 10 days showed the maximum possible modification (eye-color 5.0).

The influence of cultures of known microörganisms on eye-color modification has also been tested (medium containing 1 per cent yeast, 3 per cent sucrose, 1.5 per cent agar and 10 mg. tryptophane per 10 cc., and 3 days' growth before autoclaving). Only the unidentified *Bacillus sp.* has proved effective. *B. subtilis*, *B. mesentericus*, *B. terminalis*, *B. megatherium*, *Ps. aeruginosa*, *E. coli* and *S. cerevisiae* were without effect under the experimental conditions used. It is possible, however, that under other conditions some of these organisms might also be effective.

Discussion.—The diffusible hormones which control insect eye-color development have been considered to be specific substances produced only by insects. The results described in this report show that under suitable conditions certain bacteria synthesize a substance which has v^+ hormone activity in Drosophila. The final proof of the identity or non-identity of this active substance with v^+ hormone will be possible only when both are isolated in the pure state. However, all the available evidence indicates that the chemical and biological properties of the two substances are identical.

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NUTRITIONAL REQUIREMENTS OF DROSOPHILA MELANOGASTER[1]

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In spite of the rapid recent advance in knowledge of the nutritional and vitamin requirements of higher animals, plants and microörganisms, insect nutrition is in almost the same chaotic state as was vitamin research twenty years ago. The earlier work on insect nutrition was done before the complex nature of the B group of vitamins was known, and was often not carried out under aseptic conditions. The results obtained were therefore