DEVELOPMENT OF EYE COLORS IN DROSOPHILA: TIME OF ACTION OF BODY FLUID ON CINNABAR[1]

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THE development of vermilion eye color in mosaics of *D. simulans* is not always autonomous (STURTEVANT 1932). By transplantation experiments BEADLE and EPHRUSSI (1936) have shown that the vermilion and cinnabar eye colors of *D. melanogaster* are not autonomous in development. Vermilion and cinnabar late larval eye discs develop the wild type pigment when transplanted to late wild type larvae. Vermilion discs also develop the wild type pigmentation when implanted in cinnabar hosts, but cinnabar discs remain cinnabar in color when developing in vermilion hosts. From this and similar data BEADLE and EPHRUSSI have postulated the presence in the wild type host of two diffusible substances (v^+ and cn^+ substances), the presence of only one of these (the v^+ substance) in the cinnabar tissue, and the absence of both of these substances in the vermilion tissue.

It is of interest to ascertain when these two genetically determined diffusible substances are present and during what period of development they operate in the determination of the ultimate eye pigment. This has been done for the v^+ substance (EPHRUSSI, CLANCY, and BEADLE 1936; BEADLE, CLANCY, and EPHRUSSI 1937). They have shown by the injection of body fluid from wild type donors of various ages into apricot vermilion (w^a v) hosts 60 hours after puparium formation that the v^+ substance was not present in the wild type larvae 3 to 12 hours before puparium formation, but was present in the wild type from 3 to 80 hours after puparium formation. Similarly, body fluid obtained from wild type donors 60 hours after puparium formation and injected into apricot vermilion hosts of various ages effected a change in eye color in all hosts from late larvae to 64 hours old pupae, with a negative or weak effect at 70 hours, and negative results in still older recipients. These results demonstrate the absence of the v^+ substance in the wild type larval body fluid and its presence during approximately four fifths of the prepupal and pupal periods. The data also indicate the possibility of inducing a change in eye color from vermilion toward wild type by the injection of wild type body fluid into w^a v hosts at any time prior to approximately 70 hours after puparium formation.

The following experiments were performed to obtain similar information regarding the cn^+ substance; that is, the time during which it is present in

[1] Work done at the Institut de Biologie Physico-Chimique, Paris.

the body fluid of the wild type, and the period during which its introduction into cinnabar individuals could cause a change in their eye color. In the work of EPHRUSSI, CLANCY, and BEADLE and in our experiments, some living cells of the wild type donor were undoubtedly introduced with its body fluid into the host. The presence of these chance living cells raised a serious question in the interpretation of the results. To answer this question we have performed additional experiments using fluid from donors killed by heat and by extremely low temperature.

MATERIALS AND METHODS

In these experiments we used the same wild type Oregon-R-c, w^a *v*, and w^a *cn* stocks which were used in the work mentioned above. The rearing of the animals and the injection technique were those employed by EPHRUSSI and BEADLE 1936, and BEADLE, CLANCY and EPHRUSSI 1937. The age of the pupae was determined from the time of puparium formation, puparia being collected at 1 hour intervals. Controls for the injected larvae consisted of uninjected sibs; controls for the injected pupae were sib pupae pricked with an empty injection needle. Such controls were used regularly to determine whether any change in the eye color of the experimental hosts had occurred. Injected or pricked pupae rarely emerge and show only a partial eclosion of the head; these individuals were removed from their cases at the normal time of emergence as adult flies. All the experiments were performed at 25°C.

EXPERIMENTAL RESULTS

Time of presence of the cn^+ substance

The first series of experiments was performed to determine the time during which the cn^+ substance was present in the body fluid of the wild type donor. Body fluid from the wild type donors of known age was injected into apricot-cinnabar larvae that were close to puparium formation. The double recessives apricot-vermilion and apricot-cinnabar have been found much more sensitive indicators of the presence of the v^+ and cn^+ substances than the simple recessives vermilion and cinnabar (BEADLE and EPHRUSSI 1936). The results are shown in table 1. The first column gives the age in hours from puparium formation of the wild type donor, the second column the number of w^a *cn* recipients whose eye color was definitely shifted toward that of the normal allelomorph of *cn*, the third the number of injected flies in which the change of eye color was questionable, the fourth column the number of unaffected individuals, and the last column the total number of larvae that survived the injection and pupation and emerged as adult imagos.

The body fluid of wild type larvae close to puparium formation produced

no change in the eye color of the w^a *cn* recipients. Apparently the cn^+ substance was not present in the body fluid of the late larvae, nor in the chance cells injected with that fluid; nor was it formed by these cells later in the host. Wild type body juice obtained during the first 70 hours fol-

TABLE 1

Effect of wild type pupal fluid injected into w^acn 3-day larvae.

AGE OF DONOR IN HOURS	EFFECT ON HOST				INTENSITY OF EFFECT
	POSITIVE	QUESTIONABLE	NEGATIVE	TOTAL	
Late larvae	0	0	12	12	
Pupae					
1:10– 2:25	3	0	3	6	2 strong, 1 slight
5:30– 6:50	2	2	0	4	1 strong, 1 slight
16:30–19:15	4	0	0	4	4 strong
25:00–41:45	4	3	3	10	1 strong, 3 slight
40:00–46:00	12	1	0	13	11 strong, 1 slight
54:00–69:45	7	1	5	13	6 strong, 1 slight
71:15–72:30	1	0	1	2	slight
78:00–82:00	3	0	9	12	slight
89:45–92:00	1	9	17	27	slight
94:45–99:15	4	1	14	19	slight

lowing puparium formation and injected into w^a *cn* larvae produced a marked change in a large proportion of the surviving individuals. Body fluid drawn from wild type donors during the last 30 hours of the pupal period produced only a slight but certain effect on the pigmentation of the recipients' eyes, and this effect was obtained in a relatively small proportion of the treated animals. The cn^+ substance was present in the wild type body fluid in a high concentration for the first 0.7 of the prepupal and pupal periods, and in a low concentration thereafter until the eclosion o the adult.

The duration of the period when the cn^+ substance is present in the wild type body fluid differs from that found for the v^+ substance (EPHRUSSI, CLANCY, and BEADLE 1936; BEADLE, CLANCY, and EPHRUSSI 1937). These authors found no indication of the presence of the v^+ substance in the wild type body fluid after 80 hours from puparium formation. However, their tests were made by injection into 60 hours old w^a *v* pupae, while we used w^a *cn* larvae in testing for the presence of the cn^+ substance. We injected body fluid from wild type pupae $94\frac{1}{2}$–$97\frac{1}{2}$ hours old (close to emergence) into w^a *v* larvae and obtained definite changes in the eye color of 9 out of 11 recipients. Apparently larval hosts are more sensitive than pupal hosts as indicators of slight amounts of the v^+ and cn^+ substances. These two substances were present in the body fluid of the wild type in-

dividuals from shortly after puparium formation until close to the time of eclosion.

Time of effective-period of the cn⁺substance

Since these two substances are present during practically the entire prepupal and pupal periods in the wild type, their effective-periods in the pupae are of considerable theoretical interest. Since we have used the wild type Oregon-R-c stock while BEADLE, CLANCY, and EPHRUSSI had used the Florida wild type stock as donors in the determination of the effective-period for the v^+ substance, we considered it advisable to re-

TABLE 2

Effect of wild type pupal fluid injected into $w^a cn$ pupae.

AGE OF HOST IN HOURS FROM PUPARIUM FORMATION	EFFECT ON HOST				INTENSITY OF EFFECT
	POSITIVE	QUESTIONABLE	NEGATIVE	TOTAL	
22:30–23:45	6	0	0	6	6 strong
50:00–51:10	8	0	1	9	2 strong, 6 slight
60:20–61:30	6	2	2	10	5 strong, 1 slight
63:30–64:45	12	0	3	15	3 strong, 9 slight
64:00–65:15	12	0	0	12	2 strong, 10 slight
65:15–66:30	10	1	4	15	4 strong, 6 slight
66:40–67:50	13	0	2	15	5 strong, 8 slight
68:00–69:45	21	0	9	30	extremely slight
71:10–73:15	11	3	9	23	extremely slight
76:00–77:00	3	0	13	16	extremely slight
89:30–90:15	9	0	0	9	extremely slight
91:00–92:00	7	0	0	7	extremely slight

examine the total effective-period of the v^+ substance. The following experiments were performed to determine, with a reasonable degree of certitude, the termination of the effective-period of the v^+ and the cn^+ substances when introduced into vermilion and cinnabar recipients. In this work it was impossible to determine the time at which the reaction began because the substance may be retained and work later. The double recessives w^a *v* and w^a *cn* were used as hosts because of their more sensitive reaction, and parallel experiments were performed simultaneously with recipients of equivalent ages. The donors were wild type sib pupae collected during the same hour for injection of their body fluid into w^a *v* and w^a *cn* hosts of the same age.

The data on the effective-period for the cn^+ substance are shown in table 2. The body fluid of wild type pupae was obtained from donors 30–50 hours after puparium formation, the major portion of the work being done with 30–40 hours old donors. The fluid was injected into w^a *cn* hosts of the indicated ages in hours from the time of puparium formation. From tables

1 and 2 it is obvious that a modification of the host eye color toward the wild type was obtained when the cn^+ substance was introduced into individuals lacking it (genotype cinnabar) at any time from the late larval period until approximately 10 hours before eclosion. But there was a sharp break in the degree of eye color change between the group of w^a *cn* pupal hosts aged 66:40 to 67:50 hours and those aged 68 to 69:45 hours. The tests from 50 to 77 hours were run simultaneously allowing comparison between the animals obtained for each of the time intervals indicated in table 2. Pupal hosts 67 hours old or younger at the time of injection

TABLE 3

Effect of wild type pupal fluid injected into $w^a v$ pupae.

AGE OF HOST IN HOURS FROM PUPARIUM FORMATION	EFFECT ON HOST				INTENSITY OF EFFECT
	POSITIVE	QUESTIONABLE	NEGATIVE	TOTAL	
50:00–51:00	6	0	0	6	6 strong
60:00–61:20	14	0	1	15	12 strong, 2 slight
64:00–65:00	9	0	0	9	5 strong, 4 slight
68:00–69:15	12	0	1	13	slight
72:00–73:10	7	0	3	10	slight
76:00–77:00	10	0	0	10	slight
88:20–89:30	11	0	0	11	extremely slight
91:00–92:00	9	0	0	9	extremely slight

showed marked changes in eye color of many of the animals; in hosts 68 hours of age or older positive effects were obtained, but in every case they were extremely weak (detectable with certainty only when the eyes of the controls and experimental animals were placed under water and compared; a definite but delicate yellow could then be distinguished in the host's eyes which was not present in the eyes of the controls). It is evident that the major reaction in the eye pigment formation involving the cn^+ substance ends about 68 hours after puparium formation.

The results for the v^+ substance are shown in table 3. Positive effects were obtained again throughout all the periods tested. Here also there was a definite change in the intensity of the effect. Hosts through 61:20 hours showed pronounced or clear effects from the injections, hosts 64–65 hours showed fewer strong effects with some individuals only slightly affected (on the whole they showed rather less effect than those 2 hours earlier); beyond 68 hours the recipients showed only weak or slight effects. There was a marked reduction in the capacity to use the introduced v^+ substance at about 65 hours after puparium formation or shortly thereafter, indicating the end of the major effective-period.

Heating and freezing experiments

Throughout the above experiments injections were made with larval and pupal fluids which undoubtedly contained many living cells. This raised a serious question which has already been noted by EPHRUSSI, CLANCY, and BEADLE: were the effects obtained from the injections due to chemical compounds present at that time in the body fluid, or to the subsequent activity of chance cells injected with the fluid? To answer this question two different experiments were performed to kill the cells of the donor prior to the injections. If an effect was then obtained by the injections it might safely be assumed that the substances under consideration were already formed and present at the time of injection.

In the first experiment wild type pupae 31 hours old were sealed in a small thin walled glass ampule and immersed in a water bath at 60°C for 30 minutes. Part of the pupae were saved as controls. Since there was no further development in these controls, it was obvious that they had been killed by subjection to 60°C for 30 minutes. The body fluid of the rest of the treated pupae was injected into w^a *v* larvae. These hosts showed marked changes in their eye color, the change in color being more than half way toward that of the normal allele. Obviously the effect was not due to subsequent activity of chance cells injected with the body fluid.

In the second series wild type pupae were placed in a small vial and immersed in liquid air at about −190°C., where they were frozen. Then the vial was removed from the liquid air and the pupae were allowed to thaw. This rapid freezing at an extremely low temperature and thawing at room temperature was repeated four times. The body fluid of these dead pupae was then injected into w^a *v* and w^a *cn* larvae. Fluid from pupae 44 hours old effected a definite change in 10 of the 11 w^a *v* hosts which emerged, and fluid from 48–73 hours old donors in 15 out of 18 individuals. Fluid from pupae 1 to 2 hours old produced effects in 8 out of 9 w^a *cn* hosts which emerged, and fluid from 43 hours old pupae caused marked changes in the eye color of 10 of the 14 emerging recipients. Since the treatment killed the cells of the donors, the v^+ and cn^+ substances must have been present at the time of the injections. The two experiments demonstrate: 1) that the substances examined in this work had been formed by the wild type donors prior to the moment their body fluid was withdrawn for injection; 2) that they were apparently stable between approximately −190°C. and +60°C. (EPHRUSSI and HARNLY, 1936).

DISCUSSION

The data demonstrate that the cn^+ substance was not present in the wild type late larvae. This substance was first formed in detectable quantities at about the time of puparium formation, its presence being evi-

denced by changes in the eye color of w^a *cn* hosts injected with body fluid taken from wild type individuals one hour after puparium formation. The concentration of the cn^+ substance was quite low at this time, the changes in eye color being definite but not marked. The concentration apparently increases rapidly and in 18 hour pupae has reached a high concentration, since the amount injected changed the host mutant eye half way or more toward the color of the normal allele. Thereafter relatively large amounts of the cn^+ substance were present in the wild type body fluid as demonstrated by the degree of change in the recipient's eye color and the proportion of individuals affected. This high concentration was maintained until approximately 70 hours after puparium formation, that is, until about 18 hours after the first appearance of eye color in the wild type pupae according to SCHULTZ (1935). The body fluid of wild type pupae 70–99 hours old produced definite but only minor changes when injected into w^a *cn* larvae, and these changes occurred in a relatively small proportion of individuals, indicating that during this period the cn^+ substance was present in a low concentration. EPHRUSSI, CLANCY, and BEADLE (1936) and BEADLE, CLANCY, and EPHRUSSI (1937) have found that the v^+ substance was not present in wild type larval body fluid, but was present in the body fluid shortly after puparium formation, reached a maximum concentration between 8 and 17 hours, and thereafter showed a gradual decrease in effective power becoming undetectable in donor pupae over 80 hours old. Repeating these experiments with injections into larvae instead of pupae, we have found that traces of the v^+ substance are present even later in the wild type body fluid. Both the v^+ and the cn^+ substances seem to appear at approximately the same moment in the development of the wild type individuals. A comparison of our results with those of EPHRUSSI, CLANCY, and BEADLE seems to indicate that there is a difference in the time of fall of the high concentration of these substances; this difference may be real or may be due to the difference in the age of the recipients in the two sets of experiments.

It is difficult to define sharply the effective period for the cn^+ substance. Wild type pupal fluid from a period known to contain a high concentration of the cn^+ substance may produce marked changes in the host's eye color when injected into late w^a *cn* larvae or into pupae until 67 hours after puparium formation. But, since the substance supplied by injection might be retained by the recipient and used later, it was impossible to determine by the technique employed the time at which this substance began to be used by the host. It may be that the inception of the effective-period coincided with the first appearance of the cn^+ substance in effective concentration shortly after puparium formation, but this point is at present undetermined. The end of the major effective-period for the cn^+ substance

clearly falls between 67 and 68 hours after puparium formation and close to the first appearance of color in the eyes of cinnabar pupae. EPHRUSSI, CLANCY, and BEADLE (1936) have found that wild type body fluid containing the v^+ substance when injected into w^a v late larvae or into pupae until about 65 hours after puparium formation may have a marked effect on the recipient's eyes. In the case of the v^+ substance the major effective-period also closes before the first appearance of eye color in the vermilion pupae.

An hypothesis has been developed from the results of the transplantation of eye discs between wild type, cinnabar, vermilion, and claret (BEADLE and EPHRUSSI 1936, BEADLE and EPHRUSSI 1937). "Such an hypothesis assumes that the ca^+, v^+, and cn^+ substances are successive products in a chain reaction. The relation of these substances can be indicated in a simple diagrammatic way as follows:

$$\rightarrow ca^+ \text{ substance} \rightarrow v^+ \text{ substance} \rightarrow cn^+ \text{ substance.}"$$

From the work reported here the v^+ and cn^+ substances were apparently formed at about the same time in the development of the wild type individuals. This would be expected if they represent a chain reaction, the formation of the cn^+ substance being dependent on the presence of the v^+ substance and formed by a reaction occurring immediately after its appearance. The apparent difference in the time of fall of the high concentration of these two substances in the wild type, if real, is also in harmony with the assumption that the v^+ substance is formed first and, in a chain reaction, the cn^+ substance appears and is used later. If the difference of two or three hours in the termination of the major effective-periods for the v^+ and the cn^+ substances in w^a v and w^a cn hosts is real it is also in complete agreement with the ca^+, v^+, cn^+ chain hypothesis.

Apparently not all of the v^+ substance is used in the formation of the cn^+ substance nor all of the latter in the subsequent formation of the wild type eye pigment since effective traces of both are still present in the pupae shortly before the emergence of the flies. It is impossible to tell at present whether or not the subsequent darkening of the adult eyes with age is due to the later utilization of the last traces of these and similar substances. It has been indicated earlier in this paper that there was some use of the substances within ten hours of eclosion of the adult w^a v and w^a cn flies. It may be that the so-called "age effects" are merely the continuation or completion of processes going on before emergence.

SUMMARY

1. The body fluid of wild type late larvae does not contain the cn^+ substance (a compound capable of modifying the eye color of the w^a cn host).

2. There is a high concentration of the cn^+ substance in the wild type body fluid from 1 to 70 hours after puparium formation, the high concentration ending about 18 hours after the first appearance of eye color in the wild type pupae. It is present in a low concentration thereafter until the emergence of the adult fly. As demonstrated by BEADLE, CLANCY, and EPHRUSSI the v^+ substance also appears in the wild type individuals shortly after puparium formation, and as shown by the present experiments, can be found in a low concentration in the body fluid as late as 94 to 97 hours after puparium formation.

3. The major effective-period of the cn^+ substance introduced into w^a *cn* hosts ends between 67 and 68 hours after puparium formation, which is close to the first appearance of color in the eyes of cinnabar pupae. As previously reported by BEADLE, CLANCY, and EPHRUSSI, and confirmed here, the major effective-period of the v^+ substance introduced into w^a *v* hosts ends about 65 hours after puparium formation or shortly thereafter, that is, before the first appearance of eye color in the vermilion pupae.

4. Experiments in which chance cells introduced with the body fluid of the wild type donors were killed by heat or freezing demonstrated: that the v^+ and cn^+ substances had been formed previous to injection into w^a *v* and w^a *cn* hosts; and that the v^+ and cn^+ substances were stable between approximately $-190°$C. and $+60°$C.

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