Development of Eye Colors in Drosophila: Extraction of the Diffusible Substances Concerned

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DEVELOPMENT OF EYE COLORS IN DROSOPHILA: EXTRACTION OF THE DIFFUSIBLE SUBSTANCES CONCERNED

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Introduction.—The development of eye color in Drosophila is known to involve special diffusible substances.[1,2] A genetically vermilion (*v*) eye will develop wild-type eye color if it is supplied with v^+ substance by transplantation or by injection of body fluid of wild type flies. Similarly a genetically cinnabar (*cn*) eye will develop the color characteristic of wild type if it is supplied with cn^+ substance. The present paper summarizes preliminary experiments made to learn something of the nature of the two substances just mentioned.

During the course of our studies, Ephrussi and Harnly[3] have shown that pupal fluid can be freed of living cells by freezing in liquid air without destroying the v^+ and cn^+ substances. Khouvine, Ephrussi and Harnly[4] have shown further that these substances can be extracted from Calliphora pupae with 95 per cent alcohol-ether mixtures and with 95 per cent alcohol but not with pure ether. They conclude that these substances are not proteins or enzymes, a conclusion confirmed by our work.

Method of Testing for the Substances.—Liquid extracts are tested for v^+ substance by injections of approximately 0.4 cubic mm. into the body cavities of apricot vermilion (w^a *v*) or vermilion brown (*v; bw*) larvae shortly prior to puparium formation. In a similar way, cn^+ substance is tested for by injections into cinnabar brown (*cn bw*) larvae. The test flies normally have practically colorless eyes, and the presence of an active substance is indicated by a darkening of the eye color. The maximum modification results in an eye color similar to apricot or brown, depending on the constitution of the test fly.

It was found desirable to measure responses in a quantitative way. The test flies were separated into groups of increasing intensities of eye color and these groups were assigned values from 0 to 5. The value 0 represents no response and 5 a response such that the color is indistinguishable from brown control flies. The mean of the values obtained in this way, using from 10 to 20 injected flies, thus gives a figure which expresses in a rough quantitative way the intensity of the reaction. It is known from Malpighian tube-transplants[5] that the response varies with the number of tubes implanted and therefore presumably with the quantity of substance available in the test animal. It is not assumed that the difference in concentrations of active substance measured by any unit difference in the arbitrary scale is the same. Indeed the results suggest that a considerably

greater increase in concentration is represented by the difference between 4 and 5 than by that between 0 and 1.

Preliminary Experiments.—Wild-type pupae of *D. melanogaster*, 24 to 60 hours after puparium formation, were crushed and centrifuged into three layers, an upper one of foam or oil, a second of clear yellowish liquid and a bottom layer of sludge made up largely of cells and pupa cases. The aqueous layer, injected into w^a *v* larvae, produced a very faint or doubtful intensification in the color of the eyes; the other layers were completely negative. On the assumption that the substances were being inactivated by oxidation, pupae were crushed under sesame oil. Fluids obtained in this way were negative in all tests. The oil layers were likewise consistently negative even when the volume of oil was kept small to avoid dilution. A chloroform extract of the pupae, taken up in sesame oil, was also negative. It appeared that the substances were not lipoid-soluble, and therefore attention was re-directed to the preparation of water extracts. It was soon found that if extracting and centrifuging were carried out in an atmosphere of nitrogen, a clear watery layer could be obtained which gave strong positive tests for both v^+ and cn^+ substances.

Extractions in Nitrogen.—The procedure finally evolved was to use a wide tube, drawn out at the base into a thin-walled capillary. At the point where the bore narrowed a slight constriction was made by thickening the glass, and on this was rested a pad of glass wool. The pupae were put into the wide tube, and a piece of narrow glass tubing, sealed at one end, but with a small hole blown in the side of the seal, was fitted into the wide tube by means of rubber sleeves. Nitrogen was passed in through this tube for some minutes, the capillary then sealed off and the pupae thoroughly crushed in the atmosphere of nitrogen. The crusher was finally disconnected from the nitrogen supply and quickly sealed off, and the whole tube was then centrifuged at about 2500 r. p. m. for 15 minutes. The glass wool acts as a coarse filter, retaining pupa cases, and a clear yellowish aqueous layer collects in the capillary.

The fluid so obtained—about 0.1 to 0.2 cc. from 300 pupae—gave positive results of a strength about 2.5 in *v; bw* and from 1.5 to 2.0 in *cn bw*. The fluid prepared in this way showed a strong precipitate of coagulable protein on heating in boiling water. Heating the vessel containing pupae in boiling water for 2–3 minutes, before crushing and extraction, however, did not affect the results. Subsequent heating of the fluid extracted from heated pupae produced no further precipitation. The substances are therefore extremely heat-stable, and hence are not to be classed with enzymes or true proteins. Further, if the juice, extracted in nitrogen from boiled pupae, was exposed to air for 24 hours and then injected, the tests showed no diminution in strength; (fresh juice, 4 flies *v; bw*, mean 2.5; 24 hours old juice, 5 flies, mean 2.5). Thus, when freed from enzymes in

this way, the v^+ substance is also stable to oxidation. It was also found that active extracts could be obtained from pupae which had been dried at 100° in the oven.

On evaporating the extract to dryness in vacuo, and extracting with acetone, practically no solids were obtained in the acetone; both tests of this extract taken up in water were negative (see table 1). The residue, on freeing from insoluble matter and taking up in water, was still highly active. The active substance is therefore insoluble in absolute acetone.

TABLE 1

PREPARATION	STRENGTH OF REACTION IN *v; bw*	IN *cn bw*
Original juice (2000 pupae in 0.5 cc.)	2.5	1.75
Evaporate; extract with acetone; test the extract	0	0
Take up residue in 0.2 cc. water and filter from bulky precipitate	2.8	2.2
Treat with 0.02 cc. 5% H_2O_2	0.6	0.5
Add charcoal and boil	0.9	0.1

Oxidation of the Substances.—Extracts made in air from pupae first boiled in water or dried at 100°C. are active. The susceptibility to oxidation by air evidently is marked only in the presence of the enzymes of the pupa. Since Dakin[6] has shown that hydrogen peroxide closely imitates many types of biological oxidation, an attempt was made to reproduce the action of the enzymes of the pupa by this reagent. To an active extract, H_2O_2 was added up to a final concentration of 0.2 per cent. The solution was then gently boiled. This treatment greatly weakened the responses to both tests (table 1). No marked difference in the susceptibility of the two substances was indicated, although numerous experiments were carried out under varying conditions with H_2O_2, especially with concentrations $^1/_{10}$ to $^1/_{20}$ of the above. It appears that the activities of both cn^+ and v^+ substances are more or less completely destroyed by peroxide.

Comparison of v^+ *and* cn^+ *Substances.*—In all tests of the aqueous extracts of pupae, the response in the test for cn^+ substance was consistently weaker than that for v^+ substance, by from 0.5 to 1 point. On the other hand, Beadle[5] finds that implantation of Malpighian tubes gives results which are very nearly equal in the two tests. The above experiments give no evidence that cn^+ substance is any more easily destroyed by oxidation than the v^+ substance. It follows either that the cn^+ substance is less easily extractable than the v^+ substance, or that it takes more of the cn^+ substance to produce an equivalent effect. Strong positive results (*cn* 3.0) obtained with hot Ringer extract of Malpighian tubes from only 15 larvae, would suggest that the bulk of the substances are present in these organs, and hence the differences are ascribable to the differences in the ease with which the two substances are extracted. This conclusion is supported by an

experiment with cold Ringer extract of wild type Malpighian tubes; this extract, in *v; bw*, gave a color-value of 2.0, but in *cn bw* only about 0.8.

Finally, proof that the two tests cannot be due to the same substance, previously adduced by transplantation experiments, was given by extracting cinnabar pupae, which should contain only the v^+ substance. About 200 pupae were heated to 100°, crushed and centrifuged in nitrogen as described above, and the clear juice injected; it gave a v^+ test of about 2.5, but the *cn* test was completely negative.

Summary.—1. The substances capable of changing vermilion and cinnabar eye color toward wild type may be obtained in cell-free extracts from wild-type pupae, or the former alone from cinnabar pupae.

2. These substances are heat-stable and water-soluble, and apparently insoluble in acetone or sesame oil. They are, therefore, presumably neither proteins nor enzymes.

3. The cn^+ substance is less readily extracted than is v^+ substance.

4. Both substances are rapidly destroyed by oxidizing enzymes in the pupa juice, or, in the absence of enzymes, by dilute solutions of H_2O_2.

5. A simple method is described for roughly determining the concentration of the substances.

[1] Ephrussi, B., and Beadle, G. W., *Bull. Biol. Fr. Belg.*, **71**, 54–74 (1937).

[2] Beadle, G. W., and Ephrussi, B., *Genetics*, **23**, 76–86 (1937).

[3] Ephrussi, B., and Harnly, M. H., *C. R. Acad. Sci., Paris*, **203**, 1028 (1936).

[4] Khouvine, Y., Ephrussi, B., and Harnly, M. H., *C. R. Acad. Sci., Paris*, **203**, 1542 (1936).

[5] Beadle, G. W., these PROCEEDINGS, **23**, 146–152 (1937).

[6] Dakin, H. D., *Oxidations and Reductions in the Animal Body*, New York (1922).

DEVELOPMENT OF EYE COLORS IN DROSOPHILA: FAT BODIES AND MALPIGHIAN TUBES AS SOURCES OF DIFFUSIBLE SUBSTANCES

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From the work of Ephrussi and Beadle (see Ephrussi and Beadle[1] and Beadle and Ephrussi[2] for summaries) it is known that three diffusible substances are involved in the production of wild type eye color in *Drosophila melanogaster*. This paper is concerned with the normal sources of two of these, v^+ and cn^+ substances. Under certain conditions of genetic constitution these two substances may be produced by eye tissue itself.[1,2] Sturtevant's studies of mosaics of *D. simulans* indicated that v^+ substance