DEVELOPMENT OF EYE COLORS IN DROSOPHILA: NATURE OF THE DIFFUSIBLE SUBSTANCES; EFFECTS OF YEAST, PEPTONES AND STARVATION ON THEIR PRODUCTION

YVONNE KHOUVINE, BORIS EPHRUSSI AND SIMON CHEVAIS

(*From the Institut de Biologie physico-chimique, Paris*)

In the course of our investigation of the chemical nature of the v^+ and cn^+ substances of *Drosophila melanogaster*[1] some phenotypic effects of the culture media have been discovered, which will be described in this paper. These phenotypic effects resemble those of the v^+ hormone and undoubtedly are related to its formation in a way which remains to be determined. Therefore we shall begin with an account of the present status of our chemical work[2] and then report three kinds of phenotypic effects, namely those produced by starvation, by yeast and by peptones. The obvious question of the causal relations of the mechanisms involved will be considered in the discussion.

Methods

In our previous work on the chemical nature of the v^+ and cn^+ substances,[2] tests for the presence of these substances in a given extract were made by means of injections into animals sensitive to the corresponding substance. In the experiments reported here we used for the first time the feeding technique of Beadle and Law (1938).

Beadle and Law have demonstrated that the substances involved in the pigment formation of the wild type fly can be efficiently supplied to sensitive hosts by feeding. If vermilion brown (*v bw*) and cinnabar brown (*cn bw*) larvae are fed on cooked wild type pupae, the flies which develop show a brown (*bw*) phenotype, or a phenotype intermediate

[1] v^+ substance: a substance capable of changing the development of a vermilion (*v*) eye in such a way that it forms pigment phenotypically like that of a wild type eye.

cn^+ substance: a substance capable of changing the development of a cinnabar (*cn*) eye in such a way that it forms pigment phenotypically like that of a wild type eye.

The hormonal nature of these substances leaving no doubt, we will call them in the text either the v^+ and cn^+ substances or hormones.

[2] Earlier accounts in: Khouvine, Ephrussi and Harnly (1936); and in Khouvine and Ephrussi (1937).

between that of *bw* and *v bw* (or *cn bw*) flies. The intensity of the effect depends on the age of the larvae at the time of the transfer: according to Beadle and Law, the best results are obtained when larvae of 60 to 85 hours after egg-laying are transferred to the food containing the active substances.

On repeating these experiments with *Calliphora* extracts (*l.c.*), known to contain the v^+ and cn^+ substances, we have found that liquid extracts added to an agar solution can be tested in a similar way.

The technique used in our feeding experiments is as follows: 1 cc. of the extract or solution to be tested is mixed with 2 cc. of an agar solution. The warm mixture is poured into a cotton-stoppered shell vial, inclined at about 15° until the gelification of the medium. Unless otherwise stated, approximately thirty larvae were grown in each vial. All the experiments were performed at 25° C.

As test animals we used either apricot vermilion (w^a *v*) and apricot cinnabar (w^a *cn*), or *v bw* and *cn bw* larvae. In standard experiments the age of these at the time of the experiment was from 66 to 90 hours (after egg-laying).[3] More accurate timing was made only in some special cases, to be mentioned separately.

Our agar solution is a 3 per cent solution in Ringer's. In some of our experiments 5 per cent glucose was added to the agar solution to promote the growth of the yeast carried by the larvae.

Our standard culture medium is prepared according to the following formula: cornmeal 13 grams; brown sugar 16 grams; agar 2 grams; water 100 cc.

It will be seen that in some cases it was desirable to compare the results of the feeding experiments with those of injection. The technique of injection used was the same as previously described (Ephrussi and Beadle, 1936).

In our tables we have adopted for the description of the observed effects on *v bw* and *cn bw* flies a numerical scale essentially similar to that of Thimann and Beadle (1937). We call flies with no effect—0, and the phenotype identical with *bw*—5. One, 2, 3, 4—indicate intermediate grades of eye color modified in the direction of *bw*. In the tables the average effect (arithmetic mean) is given.

Extraction and Purification of the v^+ and cn^+ Substances

We will consider separately the extraction of the v^+ and cn^+ substances and their purification.

[3] Unless otherwise stated, eggs were collected over approximately 24-hour periods. Slight deviations from the ages here mentioned are therefore possible.

Extraction

It has been shown (Ephrussi and Harnly, 1936; Beadle, Anderson and Maxwell, 1938; Becker and Plagge, 1937) that these substances have a wide distribution among insects and can be extracted, for example, from *Calliphora erythrocephala* larvae and pupae, which can be easily obtained in Paris. Consequently we have been able to experiment on a rather large scale.

In our earlier work we have used only the pupae and more recently both pupae and larvae. From the latter we have obtained extracts as active as the pupal extracts, although they differ somewhat in total composition.

To avoid the harmful action of oxydizing enzymes on the v^+ and cn^+ substances (referred to by Thimann and Beadle, 1937) we have used organic solvents. In our extractions we have used either of two methods, one of which gives a larger product and the other a more active product and fewer impurities although the amount of extract is considerably smaller.

Alcohol-ether Extract.—The pupae are dipped twice in liquid air for several minutes at a time. These are allowed to come to room temperature and are placed in a press. The resulting juice is shaken with twice its weight of a 1 : 1 alcohol 96 per cent-ether mixture. The supernatant liquid is decanted and the residue is pressed again. This residue with twice its weight of 85 per cent alcohol is then placed in a flask connected with a reflux condenser and subjected to boiling in a hot water bath for 20 minutes.

The liquid of the first extraction separates into two layers. The alcohol, which is the lower layer, is withdrawn and added to the second extract. The whole solution is distilled in vacuum. The remaining residue is dried with absolute alcohol and extracted with benzene. It is this residue which constitutes the *nitrogenous extract* and will be referred to later as such.

By the above method 20 grams of nitrogenous extract can be procured from 1 kilogram of pupae. This extract is highly active in 10 per cent solutions when used in the feeding test as described above, or in 1 per cent solutions when injected into the body cavity of larvae.

Carbitol Extract.—In this method the extraction was usually carried out on a mixture of pupae and larvae. The animals are crushed and dipped in three times their weight of acetone. The acetone is decanted and the residue is carefully pressed. This is crushed again and dipped in enough carbitol to cover it. It is then incubated at 29° for 18 hours, decanted and pressed. The resulting solution is yellowish brown. To this solution an equal volume of chloroform is added. If the liquid

does not separate into two layers, a little water is added (however, the latter is usually unnecessary). The chloroform-carbitol layer is withdrawn and washed two or three times with water. The aqueous solutions, which still contain a little carbitol, are combined and distilled in a vacuum. The active substances are precipitated out of the remaining solution by means of ether or acetone.

In this manner we obtain what we shall name the *carbitol extract.* Six kilograms of larvae produce about 20 grams of this extract. In 0.5 per cent solutions it produces very strong effects on *v bw* flies (feeding test) and much weaker effects on *cn bw* flies. It seems that the carbitol extracts v^+ substance more nearly completely than it does cn^+ substance.

Purification of the Extracts

Purification of the Nitrogenous Extract.—A solution of nitrogenous extract is first precipitated by means of basic lead acetate; the precipitate is removed and H_2S is passed through the solution (which contains most of the substances) to remove the excess lead. The liquid is acidified with H_2SO_4 and an excess of a phosphotungstic acid solution is added. The solution is filtered and it has been found that the phosphotungstic filtrate does not contain the v^+ and cn^+ substances. The precipitate is treated with baryta and the baryta solution is found to contain these substances. By fractionating this solution with $AgNO_3$ first in acid, then in alkaline medium, the active substances are carried with the purines, the histidine, and even with the arginine.

Attempts to purify further by means of flavianic acid, picric acid, and picrolonic acid were unsuccessful, because neither the precipitate nor the filtrate were active. Apparently, the latter reagents act on the substances at this stage in a destructive manner. Reinecke salt precipitates have also been found inactive.

The substances extracted from the phosphotungstic precipitate produce weak but definite effects in a 1 per cent solution. Thus by means of this purification the activity was increased tenfold, although a large quantity of the substance was lost in the manipulation.

Purification of the Carbitol Extract.—The above-mentioned ether precipitate is dissolved in water acidified with H_2SO_4. To this, phosphotungstic acid is added until no further precipitate is formed. This is placed in the refrigerator for several hours. The precipitate is then dissolved in baryta, in which the active substances are soluble. This solution is neutralized by means of CO_2 and distilled in a vacuum. The dry residue obtained is reddish brown and is definitely active in 0.25

per cent solutions. Here again, further attempts to purify by means of picrolonic acid or the Reinecke salt have been unsuccessful.

It follows, therefore, from the above data that the v^+ and cn^+ substances are neither enzymes nor proteids. Furthermore, they are not polysaccharides, since the products of their hydrolysis do not reduce Fehling's solution. They seem to belong to the group of amino bases, since they are precipitated by phosphotungstic acid. The above conclusions are in full agreement with those of Thimann and Beadle (1937), and Tatum and Beadle (in press).

Concentration of Extracts and Intensity of Effects

Beadle (1937) has shown that wild type Malpighian tubes produce and release the v^+ and cn^+ substances. The implantation of one, two, three or four wild type Malpighian tubes into *v bw* and *cn bw* hosts has shown that the effects produced are, roughly speaking, proportional to the amount of implants.

The feeding technique offers the possibility of testing such relations in a different way. Table I gives the results of an experiment in which

Table I

Relation between concentration of extract and intensity of effect

Concentration of extract	*v bw*			*cn bw*		
	Number of hatched flies	Number of modified flies	Intensity of effect	Number of hatched flies	Number of modified flies	Intensity of effect
per cent						
0.1	8	5	0.8	4	0	0
0.5	3	3	2.0	3	3	2.0
2.5	23	23	3.0	22	22	3.3
5.0	23	23	4.5	23	23	4.0
12.5	7	7	5.0	11	11	5.0
25.0	16	16	5.0	16	16	5.0
50.0	10	10	5.0	8	8	5.0

v bw and *cn bw* larvae have been fed on various concentrations of *Calliphora* nitrogenous extract. The medium used consisted of 2 cc. agar + 1 cc. of extract of which the concentration is given in the first column of the table.

It may be seen that, at low concentrations (0.1–12.5 per cent) the effect increases with the concentration of the extract. At 12.5 per cent the maximum effect ($5 = bw$) is obtained. This maximum is obviously reached by the *v bw* and *cn bw* flies at similar concentrations.

It should be noticed also that, except in the lowest concentration, effects are produced in 100 per cent of the flies.

The lowest concentration producing a detectable effect seems to be lower for *v bw* than for *cn bw* flies. Table II gives the results of a

TABLE II

Relation between concentration of extract and intensity of effect on v bw and cn bw

Concentration of extract	*v bw*		*cn bw*	
	Number of flies	Intensity of effect	Number of flies	Intensity of effect
per cent				
0.05	8	0	10	0
0.1	8	1.1	14	0
0.25	8	2.0	15	1.0
0.5	10	2.2	19	1.8
1.0	27	2.6	17	2.0

more detailed experiment on the action of low concentrations, which corroborates this conclusion.

Experiments similar to the above have been made with agar containing glucose as well as with agar containing no glucose and with larvae carefully washed to remove the adherent yeast. The experiments gave essentially similar results. On the other hand, strong effects can be produced by adding the nitrogenous extract to the standard cornmeal medium.

EFFECT OF STARVATION

In testing the effects of some chemicals by the feeding method, it was found that some of the control animals, grown on the agar glucose mixture, showed a slight effect similar to that of the v^+ hormone. For example, in one experiment, thirty w^a *v* larvae were removed from the standard culture medium 72–96 hours after egg-laying and placed in a vial containing 2 cc. of a 3 per cent agar solution in Ringer's containing 0.5 per cent glucose. Out of 15 flies hatched in this vial, 12 hatched on the 6th day; the phenotype of these flies was normal, but the size of half of them was clearly smaller than that of the controls. Three flies hatched on the next day: one of these, although small, was normal again, and two showed an eye color clearly modified in the direction of the w^a phenotype; their size was again below the average of the control animals.

The experiment was repeated with w^a *v*, w^a *cn*, *v bw* and *cn bw* larvae and gave essentially similar results for the vermilion flies, while no effect whatsoever was observed on the *cn* flies. The experiment,

later repeated on a large scale, confirmed this difference between vermilion and cinnabar flies.

The question was raised as to what part the agar or the glucose played in the production of the observed effects. Two kinds of experiments were performed: *v bw* larvae were grown either (1) on glucose-free agar, or (2) on cotton moistened with Ringer's containing 3 per cent glucose. The two experiments gave similar results: still smaller percentages of flies reached the age of hatching and among these a small number showed a modified eye color.

Special attention is drawn to the fact that only a relatively small percentage of animals showed the modification of eye color and that the flies hatched first, never showed this modification. This at once suggested that the time at which the larvae are removed from the normal food is of importance for the outcome of the experiment. This view was confirmed by experiments in which younger larvae were transferred to the agar medium. For instance, in one experiment, *v bw* larvae collected on two consecutive days were used. The age of the larvae of the first batch, at the time of the experiment, varied between 48 and 72 hours, that of the second batch between 72 and 96 hours (after egg-laying). The results of this experiment are given in Table III. As seen from the table, this experiment has shown a higher

TABLE III

Relation between age of larvae and frequency of starvation effect

	Number of larvae used	Total of hatched flies	*v bw* phenotype	Modified phenotype	Percentage modified
Young larvae.....	240	29	11	18	62
Old larvae.....	240	121	82	39	32

percentage of modified flies when younger larvae were treated; but the total number of flies was not modified. This suggested that the age of the larvae was not the only factor involved.

At the time these results were obtained, it was learned that Dr. Beadle and his co-workers were concerned with a general study of the effects of starvation on the development of *Drosophila*. Our data, which needed further and more precise elaboration, were therefore communicated to Dr. Beadle. The paper immediately following this summarizes the experiments made by Beadle, Tatum and Clancy.

From the above results we conclude that undoubtedly the effects observed are due to starvation. The influence of the age of the larvae on the frequency of the effect, the variability of the size of the flies,

the absence of any definite constituent of the medium to which the effect could be attributed—all point to this interpretation.

Effect of Yeast

In the course of our chemical work, when using the feeding technique, we sometimes met with the following difficulty. The fractions to be tested, mixed with agar, were often not nutritive enough to cover the developmental needs of the larvae. To overcome this difficulty, we tried to add to our media small amounts of dry yeast. Some experiments of this kind brought up an unexpected result: *v bw* flies developed with a strongly modified phenotype (modification of the eye color in the direction of the *bw* phenotype), under conditions where the effect could be ascribed only to the dry yeast.

Experiments performed for the purpose of ascertaining the nature of this relation failed at first or gave irregular results, indicating the operation of an unknown factor. Experiments have been devised then to test the possible influence of the concentration of the dry yeast. The results of these have explained the reasons for the previous inconsistency by showing that the effect, after reaching a maximum at a certain (relatively low) concentration, very rapidly decreases and falls

Table IV

Effect of different concentrations of dry yeast on v bw and cn bw

Dry yeast	*v bw*			*cn bw*	
	Number of hatched flies	Number of modified flies	Intensity of effect	Number of flies	Intensity of effect
mg.					
10	9	9	3.0	4	0
15	20	20	3.3	21	0
30	26	26	3.5	21	0
60	25	25	1.7	27	0
125	21	13	0.8	19	0
250	15	0	0	23	0

to zero. Table IV gives the results of one of the experiments of this type (medium: 4 cc. agar + the indicated weight of powdered dry yeast). It can be seen, moreover, that while strong effects are produced on the eye color of *v bw* flies, *cn bw* show no modification whatsoever.

Table V gives the results of two parallel experiments on *v bw* flies, in one of which the indicated amount of dry yeast was added to 4 cc. of the agar-glucose mixture and, in the other, to 4 cc. of glucose-free

agar. In the latter case the larvae, before being placed on the medium, have been carefully washed in alcohol and Ringer's to remove the adherent yeast.

It is seen that the effects are weaker in the presence of glucose. We shall return later to the question as to whether this inhibition is due to the glucose contained in the medium or to the growing yeast. In addition to the foregoing, experiments have been made with a nitrogenous extract of dry yeast, prepared in a manner similar to that of the nitrogenous extracts of *Calliphora*. Among these we will quote only one experiment, in which *v bw* larvae were grown on 2 cc. of agar (with or without glucose) + 1 cc. of extract of the concentration indicated in Table VI. Moreover, two vials of each of the media were prepared:

TABLE V

Action of glucose on the intensity of the effect of dry yeast

Dry yeast	With glucose			Without glucose		
	Number of hatched flies	Number of modified flies	Intensity of effect	Number of hatched flies	Number of modified flies	Intensity of effect
mg.						
10	19	14	1.5	2	0	0
15	22	21	1.9	2	2	3.3
30	23	19	1.6	17	17	2.6
60	29	9	0.3	24	24	1.7
125	35	3	0.2	23	23	0.3
250	29	0	0	22	13	0.5

in one of them carefully washed larvae were placed, and into the other the larvae were transferred directly from the standard cornmeal medium (Table VI).

We find here again that the effect increases with the concentration up to a maximum (at 2.5–5.0 per cent in this experiment) and then decreases and disappears completely; and that the effect is almost entirely suppressed in the two sets of vials containing glucose, while there is no difference between the two sets of vials containing glucose-free agar (washed and not-washed larvae). It therefore seems probable that the inhibition of the effect in the agar-glucose mixture is due to the glucose and not the growing yeast cells.

Experiments consisting of feeding *cn bw* larvae on different concentrations of dry yeast gave consistently negative results.

TABLE VI

Effect of nitrogenous extract of dry yeast on v bw in glucose-containing and glucose-free agar

Concentration of extract	Without glucose				With glucose			
	Washed		Not washed		Washed		Not washed	
	Number of flies	Effect	Number of flies	Effect	Number of flies	Effect	Number of flies	Effect
per cent								
0.25	1	0	3	0.8	14	0.1	15	0
0.5	2	0	7	0.4	20	0	18	0
1.0	14	1.4	5	0.8	18	0.3	20	0.02
2.5	20	2.1	19	2.3	21	0.1	20	0.2
5.0	16	2.6	18	2.0	16	0	20	0
12.5	17	0.9	22	0.6	21	0	20	0
25.0	14	0	20	0	21	0	22	0

Table VII summarizes the results of injection of dry yeast nitrogenous extract (dissolved in Ringer's) into *v bw* larvae. No modification of the eye color has been observed.

Since growing yeast cells are the normal food of *Drosophila,* the question was raised whether the " activity " of the dry yeast depends on

TABLE VII

Injections of nitrogenous extract of dry yeast

Concentration of extract (*per cent*)	Number of flies	Effect
0.1	11	0
0.5	7	0
2.5	12	0

its method of preparation. Two attempts to answer this question have been made.

A nitrogenous extract of *fresh* yeast has been prepared. Fed to *v bw* larvae this extract has shown a fair activity (2.3) with a maximum at 2.5 per cent and a decrease above this concentration.

On the other hand, fresh yeast has been killed by freezing it twice in liquid air and melting at 38° C. Mixed with agar this yeast produced very clear effects (3.0) and also with a characteristic maximum at the concentration of 1 drop per 2 cc. agar.

From the last two experiments it cannot be concluded whether or not the living yeast cells have the same properties as the dry yeast or yeast cells killed by freezing. The question as to why the growing yeast does not produce any effect on *v* flies under the normal culture conditions cannot be answered at present. We will only mention several possible explanations: the effects described here might be produced by a property due to the preparation methods (killing, drying, extraction). On the other hand, it is possible that no effects are usually produced because the larvae ingest too high a dose of yeast. And finally the absence of effect might be due to the high sugar content of the standard culture media.

Effect of Peptones

An experiment in which thirty *v bw* and thirty *cn bw* larvae have been raised on a mixture of 2 cc. of the agar-glucose solution + 1 cc. of a 10 per cent solution of peptone (Chapoteaut), has shown that this peptone produces an effect similar to that of the v^+ hormone. Out of 21 *v bw* flies hatched, 17 showed rather weak, but definite effects (average value — 1.8). The 23 hatched *cn bw* flies did not show any modification of the eye color.

The experiment was repeated with various concentrations of peptone, the results of which are shown in Table VIII. It can be seen that, like

Table VIII

Effect of various concentrations of peptone on v bw

Concentration of peptone (*per cent*)	Number of flies	Intensity of effect
1	24	0
2	25	0
4	18	0.1
8	27	1.4
16	25	1.6

yeast, the peptone produces an effect increasing with the concentration.

In the experiment given in Table IX higher concentrations of peptone have been tested. The data show that, after having reached a maximum (at 10 per cent in this experiment) the effect decreases again: here it falls to 0 at 50 per cent, i.e. the decrease is much slower than for the yeast extract.

TABLE IX

Relation between concentration of peptone and intensity of effect on v bw

Concentration of peptone	Number of flies hatched	Number of modified flies	Intensity of effect
per cent			
1	4	0	0
5	23	23	1.6
10	25	25	2.2
20	29	29	1.4
30	28	23	0.9
40	33	18	0.4
50	25	5	0.2

Table X gives the results of another experiment, in which 1 cc. of a 10 per cent or 20 per cent peptone solution was added either to the glucose agar mixture or to the standard cornmeal medium (test larvae

TABLE X

Comparison of the effects of peptone in agar and in cornmeal media

Concentration of peptone	In agar		In cornmeal	
	Number of flies	Intensity of effect	Number of flies	Intensity of effect
per cent				
10	22	2.2	29	0
20	26	1.7	33	0

—*v bw*). It is seen that the effect of peptone is suppressed in the cornmeal medium.

Table XI gives the results of the comparison between three different peptones and proteoses (1 cc. of a 10 per cent solution + 2 cc. of glucose-agar; test animals—*v bw*). It appears that Witte peptone,

TABLE XI

Effects of various peptones and proteoses on v bw

Substance	Number of flies	Intensity of effect
Witte peptone 10%	37	2.3
Gelatin peptone 10%	35	0
Fibrin peptone 10%	37	2.1
Proteoses 10%	34	1.3

fibrin peptone and proteoses produce clear effects, while gelatin peptone does not.

It is known that gelatin peptone differs from the other peptones tested by the lack of tyrosine and tryptophane. We tested therefore on *v bw* larvae the action of these two substances alone or in combination with gelatin peptone (Table XII). The data clearly show that the

TABLE XII

Complementary effect of tryptophane

	Number of hatched flies	Number of modified flies	Intensity of effect
Tyrosine 1%	11	0	0
Tryptophane 1%	12	1	0.1
Tyrosine 1%+tryptophane 1%	10	0	0
Gelatin peptone 10%	25	0	0
Gelatin peptone 10%+tyrosine 1%	25	0	0
Gelatin peptone 10%+tryptophane 1%	18	15	2.5
Gelatin peptone 10%+tyrosine 1%+tryptophane 1%	21	17	2.0

mixture gelatin peptone-tryptophane produces a considerable effect. The tryptophane itself does not modify the eye color: out of 12 flies hatched in this vial only one showed an effect which we attribute to starvation.

In addition to the above experiments, injections of the active mixtures into *v bw* larvae have been made. Thirty larvae were injected with a 2 per cent solution of Chapoteaut peptone; out of these four flies hatched showing no modification of eye color. Again thirty larvae were injected with a 1 per cent gelatin peptone + 0.5 per cent tryptophane mixture, out of which the eight hatched flies showed no modification of the eye color.

MIXTURES OF EXTRACTS

For the comparison of the different effects studied it was of interest to see whether the "inhibiting" effect of the high concentrations of dry yeast extract will suppress the effects of active concentrations of *Calliphora* extracts or of the peptone. The results of two such experiments with *v bw* larvae are given in Table XIII. It is clear that the yeast extract has not modified the effect of a 5 per cent *Calliphora* extract, while it has completely suppressed the effect of 10 per cent peptone.

TABLE XIII

Effect of addition of dry yeast extract to Calliphora extract and to peptone

	Number of flies	Intensity of effect
Dry yeast extract 25%	33	0
Calliphora extract 5%	31	3.0
∫Dry yeast extract 25% *Calliphora* extract 5%	39	3.0
Peptone (Chapoteaut) 10%	39	1.5
∫Dry yeast extract 10% \Peptone (Chapoteaut) 10%	38	0

TIME RELATIONS IN THE EFFECTS STUDIED

In describing the feeding technique we have mentioned that, according to Beadle and Law, the effect produced on *v bw* and *cn bw* by feeding them on crushed wild type pupae depends on the age of the larvae at which this feeding is begun. Curve A in Fig. 1 gives the results of Beadle and Law for *v bw* larvae. In this curve the intensity of the effect is plotted against the age (from hatching of the eggs) at which the larvae are transferred from the standard culture medium to the tested medium. The curve of Beadle and Law shows then that the strongest effects are obtained when larvae are grown on the standard food up to 36–60 hours after hatching [5] and then transferred to the crushed wild type pupae. If transferred before or after this time, only very weak effects are produced.

The question was raised whether this time relation, characteristic of the v^+ hormone, may be found in producing similar effects by means of the dry yeast extract or the peptone. An experiment was performed in which larvae timed at hatching and grown on the cornmeal medium were transferred at various known ages to the following three media: (1) 2 cc. agar with glucose + 1 cc. of a 10 per cent *Calliphora* nitrogenous extract; (2) 2 cc. agar without glucose + 1 cc. of a 2.5 per cent dry yeast nitrogenous extract; (3) 2 cc. agar with glucose + 1 cc. of a 10 per cent gelatin peptone solution containing 1 per cent tryptophane. The results of these three experiments are given in Table XIV and Fig. 1 (curves 1, 2 and 3). We should like to emphasize that we do not ascribe to this figure any rigorous quantitative value; we merely reproduce it to facilitate the comparison of the different results.

[5] In the paper of Beadle and Law the ages of the larvae are indicated in hours from egg-laying. The curve represented on our figure is obtained by subtracting 24 hours from the ages given by Beadle and Law. After our manuscript had been sent to press Dr. Beadle informed us that according to his determinations hatching occurs 18 hours after egg-laying. Consequently curve *A* should be shifted 6 hours to the left.

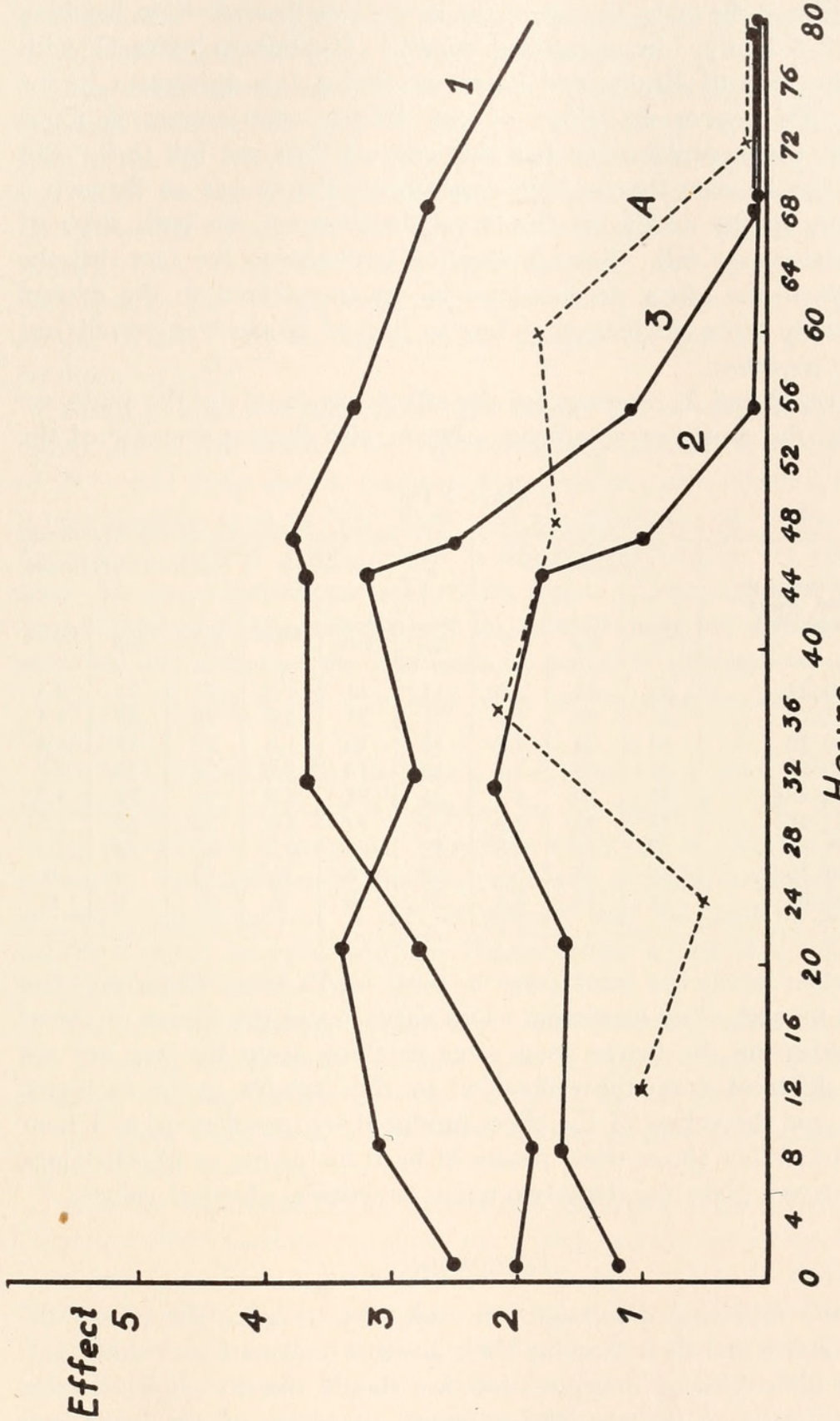

FIG. 1. Relation between effect on eye color and time of transfer of larvae (hours after hatching of eggs) from standard culture medium to the experimental medium. Curve A is based on data of Beadle and Law (1938).

As seen in the table, the age of the larvae was determined at hatching within 2–3 hours. In comparing curve 1 (*Calliphora* extract) with curve A (data of Beadle and Law) we find a fair agreement in the results. Of course the effect of our 10 per cent extract is much stronger, which explains the fact that curve 1 does not fall to 0. But then it can be seen that in both experiments the curves go through a maximum in the middle of the larval development, on both sides of which the curves fall. Special attention is drawn to the fact that the intensity of the effect produced on larvae transferred to the extract immediately after hatching is as low as that of larvae transferred just prior to pupation.

Curves 2 and 3, representing the effects produced by the yeast extract and the peptone-tryptophane solution, also show a decrease of the

TABLE XIV

Age of larvae at transfer (hours from eclosion)	*Calliphora* extr.			Dry yeast extract			Peptone+tryptophane		
	Total flies	Modified	Effect	Total flies	Modified	Effect	Total flies	Modified	Effect
0–2½	25	25	2.0	12	11	1.2	22	21	2.5
7–10	34	34	1.9	21	21	1.7	25	25	3.1
20–22	33	33	2.8	26	26	1.6	32	32	3.4
30–33	35	35	3.7	18	18	2.2	30	30	2.8
43½–45½	29	29	3.7	25	24	1.9	21	21	3.2
45½–48½	24	24	3.8	27	17	1.0	24	23	2.5
54–57	23	23	3.3	27	3	0.1	27	12	1.1
66–70	26	26	2.7	29	0	0	27	0	0
78½–82	25	23	1.9	27	0	0	27	0	0

effect when larvae are transferred to these media after 46 hours. But there is no such clear maximum as on curve 1 and the effects produced by transferring the larvae soon after hatching from the egg are not clearly different from those obtained by the transfer at 32–46 hours. In any case the values of the effect produced by transferring at 1 hour are considerably above those produced by transferring at 68–80 hours, while curve 1 gives for these two points practically identical values.

DISCUSSION

In the following discussion we shall first compare the effects described above and then examine their possible explanations. Anticipating the difficulties of interpretation, we should like to emphasize that we are aware of the speculative nature of many of the arguments presented, due to the incompleteness of information in the present stage

of the work. Nevertheless, a detailed discussion is judged desirable, if only for tracing the lines of new experiments.

The common characteristic of all the effects described is that they simulate the effect of the v^+ hormone. There is no reason to assume that, in the last analysis, the effects are produced by anything else than this hormone, but it is clear also that the effects of yeast and peptone (let alone starvation) are not due to the presence of hormone in these media: it has been shown that neither dry yeast, nor dry yeast extract, nor peptones produce any detectable effects when injected into the lymph of the larvae. This is, however, the crucial test for the presence of hormone as such.

Putting aside the starvation effect which is not necessarily due to the nature of the medium, we may say that the effects of peptones and of dry yeast have also in common their relation to concentration: the proportionality to it at low doses and the inhibition at higher doses. Since the inhibition is shown both by the peptone and by dry yeast and since the yeast extract can inhibit the action of the peptones, it is at least highly probable that there is no special inhibiting substance and that, on the contrary, the inhibition is bound to the active principle (whatever its nature is) contained in, or represented by, the yeast and the peptone.

It has been shown that the action of dry yeast (or yeast extracts) is inhibited by the presence of glucose in the medium. On the other hand, the effects of peptone have been observed in the glucose-agar mixture. This difference between yeast and peptone might, however, be purely quantitative. It will be noticed that the peptone action is inhibited in the cornmeal medium, characterized among other things by a higher sugar-content.

Both the peptone and yeast effects are dependent upon the age of the larvae to which they are fed. The age-effect curves are similar, although different somewhat from the curve for hormone action.

All these facts show a close resemblance between the action of the peptones and dry yeast.

Another characteristic feature of the facts described is that all the factors studied produce an effect on *v* flies and no effect on *cn* flies. This fact is a new argument in favor of the notion of Beadle and Ephrussi (1936, 1937) of the non-identity of the v^+ and cn^+ hormones. The argument is further strengthened by the fact that carbitol extracts seem to produce much stronger effects on *v*, while the nitrogenous extract produces the maximum effect at quite similar concentrations in *v* and *cn* flies.

Turning to the interpretations of the observed facts, let us first consider the possible mechanism of the starvation effect. Starvation brings about a change of eye color which is similar to that produced by the v^+ hormone. We might as well then assume that under the conditions of starvation an amount of the hormone sufficient to produce a visible effect is produced by v flies.[6] The question is then raised as to the origin of this hormone. Three different hypotheses might be suggested:

(*a*) We can assume that v flies normally produce some v^+ substance and that under the conditions of starvation the process of hormone production is accelerated more or slowed down less than other developmental reactions.

(*b*) We can assume also that v flies normally possess all the necessary elements required in the hormone formation, but that normally their metabolism is directed on a track not leading to the production of hormone. On this assumption the formation of v^+ hormone would result from the breakdown or resynthesis of some normal constituents of the tissues and, in v animals, would occur only under the conditions of starvation, i.e. they would represent a deviation from the normal metabolism.

(*c*) Finally, we might try to explain the starvation effect in terms of the quantity of food ingested. We have seen that dry yeast produces a similar effect when supplied in small amounts and that this effect is not observed when the animal is supplied with larger amounts of yeast. Assuming that living yeast has the same properties (an assumption which is by no means demonstrated) we might interpret the starvation effects as due to the ingestion of a small ("active") dose of yeast.

We will note that interpretations (*b*) and (*c*) do not imply either the complete lack, or, on the contrary, a small production of v^+ substance by v animals.

To distinguish between these three hypotheses is a rather difficult problem.

Although Beadle and Ephrussi (1937) pointed out that "from the nature of the tests used for such diffusible substances, it is obvious that 'absence' can mean only a quantity as small as or smaller than that produced by the test mutants," hypothesis (*a*) does not seem very probable on several grounds. It has been shown by Beadle, Clancy and Ephrussi (1937) that injection of v lymph into sensitive animals does not lead to a modification of eye color of the injected animals. Beadle (1937) has shown by injecting concentrated extracts of Malpighian

[6] According to a personal communication of Dr. G. W. Beadle the v^+ hormone can actually be extracted from starved v larvae.

tubes of *v* larvae (wild type Malpighian tubes do produce both the v^+ and cn^+ substances) that the amount of v^+ substance in *v* larvae is not more than about one-ninetieth of the amount present in wild type (see also Beadle, Tatum and Clancy in the following paper concerning this). We should expect then that to produce an accumulation of a considerable quantity of the so slowly produced hormone must require a very serious retardation of the development. Although small retardations always accompany a clear starvation effect, they do not appear as sufficient to account for this effect. (Precise determinations and calculations will be found in the paper by Beadle, Tatum and Clancy.)

Hypothesis (*c*) is equally hard to disprove. We will only mention here that, according to a personal communication of Beadle, the starvation effect can be observed on animals starved during a very short (sensitive) period. The total amount of yeast taken in is then very close to the normal one.

In spite of this uncertainty we favor hypothesis (*b*), which appears to us considerably supported by the well-known fact that, during starvation, the flies live mainly on their own fat bodies (Guyenot, 1917).

For the explanation of the effects of the dry yeast and of the peptones only two hypotheses remain possible, since we have already discarded the possibility that they contain the v^+ hormone:

(1) We can assume that they contain a specific substance.

(2) We can interpret their effects in terms of starvation.

It has been shown that both the dry yeast (or yeast extracts) and the peptones produce their characteristic effects only at low concentration. One might assume then that this low concentration represents a low food level and that what we referred to as inhibiting action of high concentrations is nothing but a normal nutrition. Such an interpretation, difficult as it is to discard in the case of the yeast effects, is not in agreement, however, with the observation that yeast cells grow perfectly on the peptone media used. We have to discard this hypothesis at least in so far as the peptone effect is concerned and to give our preference to hypothesis (1).

Concerning the assumption that the peptone and, possibly, dry yeast contain a specific substance, we have to make two remarks:

(1) This substance might act on the larva either directly or through the intermediary of the growing yeast. The latter interpretation does not fit, however, the effects of yeast extracts, since here the effects are observed in glucose-free media and on larvae washed to remove the adherent yeast (i.e. in the absence of growing yeast).

(2) Concerning the rôle of the substance or, more probably, substances (in view of the complementary action of tryptophane) we are,

at first sight, again in the presence of two somewhat distinct possibilities: the substances might represent normal constituents of the hormone or, on the contrary, they might have nothing to do with the hormone, except that, when taken in with the food, they change the normal metabolism of the larva in a way similar to that produced during starvation. No decisive argument for or against one of these possibilities can be advanced at present.

No conclusion as to this question can be drawn from the experiments with dry yeast extracts: while their close relation with the normal food of *Drosophila* at first sight does not incline us to think of the yeast effects in terms of a modification of metabolism, the literature contains many indications of clear biochemical differences between living and dry yeast. On the other hand, the inhibition of the yeast effect by glucose offers a striking analogy with the phenomenon of protein-sparing.

When one comes to think of the described phenomena and of the different suggested hypotheses in these terms, it becomes obvious that the different interpretations might be more closely related than they appear to be at first sight. Not only the distinction between them becomes more and more subtle, but a situation becomes perfectly conceivable in which a specific substance would produce a definite effect only in the presence of a specific deficiency. To say that an effect produced under such circumstances is due to a specific substance or to a modification of the metabolism, becomes a matter of taste. Thus, the consideration of the effects of yeast and peptone lead us to notions essentially similar to that to which we have been led by the consideration of the starvation effect. The attack of the fat bodies during starvation, the importance of tryptophane in the effect of peptones, the inhibition of the yeast effect by glucose, the conclusion of our chemical work indicating the probable amino-base nature of the v^+ substance—all point to the protein breakdown as source of this hormone.

To take in further work such an intermediate position has the advantage of drawing one's attention to the common background of the different phenomena: that of the yeast effect, that of the effect of peptones and even that of the effect of starvation.

Summary

An account of chemical investigations of the nature of the v^+ and cn^+ hormones of *Drosophila* is given. It is concluded that they probably belong to the group of amino-bases.

It is shown that the effects of *Calliphora* extracts, containing these hormones, are proportional to the concentration.

It is shown that under the influence of starvation *v* flies undergo a modification of the eye color, similar to that produced by the v^+ hormone.

It is shown that similar effects are produced by feeding *Drosophila* larvae on small doses of dry yeast. At low doses the effects produced are proportional to the concentration of yeast. Higher doses of dry yeast suppress the effect which is also suppressed by the presence of glucose in the medium.

Various peptones also produce a hormone-like effect when fed to *v* larvae. Again the effect is proportional to the concentration of peptone at low concentrations, and decreases above a certain maximum.

High concentrations of yeast extract suppress the effects of the peptones, but not the effect of *Calliphora* extracts.

The relations of the age of the larvae to the intensity of the effects produced by yeast extract and by peptone are studied.

Gelatin peptone does not produce an effect comparable to that of the other peptones tested, but does if tryptophane is added.

Neither starvation, nor dry yeast, nor peptones, produce the modification of the eye color of *cn* flies.

Neither dry yeast, nor peptones produce effects on *v* flies when injected into the body cavity of the larvae.

An attempt is made to interpret all the effects observed by a single hypothesis.

LITERATURE CITED

BEADLE, G. W., 1937. Development of eye colors in Drosophila: fat bodies and Malpighian tubes in relation to diffusible substances. Genetics, **22**: 587–611.

BEADLE, G. W., R. L. ANDERSON, AND JANE MAXWELL, 1938. A comparison of the diffusible substances concerned with eye color development in Drosophila, Ephestia and Habrobracon. *Proc. Nat. Acad. Sci.*, **24**: 80–85.

BEADLE, G. W., C. W. CLANCY, AND BORIS EPHRUSSI, 1937. Development of eye colours in Drosophila: pupal transplants and the influence of body fluid on vermilion. *Proc. Roy. Soc., B,* **122**: 98–105.

BEADLE, G. W., AND BORIS EPHRUSSI, 1936. The differentiation of eye pigments in Drosophila as studied by transplantation. *Genetics,* **21**: 225–247.

1937. Development of eye colors in Drosophila: diffusible substances and their interrelations. *Genetics,* **22**: 76–86.

BEADLE, G. W., AND L. W. LAW, 1938. Influence on eye color of feeding diffusible substances to Drosophila melanogaster. *Proc. Soc. Exper. Biol. and Med.,* **37**: 621–623.

BEADLE, G. W., E. L. TATUM, AND C. W. CLANCY, 1938. Food-level in relation to rate of development and eye pigmentation in Drosophila melanogaster. *Biol. Bull.,* **75**: 000.

BECKER, ERICH, AND ERNST PLAGGE, 1937. Vergleich der die Augenausfärbung bedingenden Gen-Wirkstoffe von Ephestia und Drosophila. *Naturwiss.,* **25**: 809.

EPHRUSSI, BORIS, AND G. W. BEADLE, 1936. A technique of transplantation for Drosophila. *Am. Nat.,* **70**: 218–225.

EPHRUSSI, BORIS, AND MORRIS H. HARNLY, 1936. Sur la présence, chez différents Insectes, des substances intervenant dans la pigmentation des yeux de Drosophila melanogaster. *Compt. Rend. Acad. Sci. Paris,* **203**: 1028–1030.

GUYENOT, E., 1917. Recherches sur la vie aseptique et le développement d'un organisme en fonction du milieu. Thesis, Paris. 330 pp.

KHOUVINE, YVONNE, BORIS EPHRUSSI, AND MORRIS H. HARNLY, 1936. Extraction et solubilité des substances intervenant dans la pigmentation des yeux de Drosophila melanogaster. *Compt. Rend. Acad. Sci. Paris,* **203**: 1542.

KHOUVINE, YVONNE, AND BORIS EPHRUSSI, 1938. Fractionnement des substances qui interviennent dans la pigmentation des yeux de Drosophila melanogaster. *Compt. Rend. Soc. Biol.,* **124**: 885–887.

TATUM, E. L., AND G. W. BEADLE. Development of eye colors in Drosophila: Some properties of the hormones concerned. *Jour. Gen. Physiol.* In press.

THIMANN, KENNETH V., AND G. W. BEADLE, 1937. Development of eye colors in Drosophila: extraction of the diffusible substances concerned. *Proc. Nat. Acad. Sci.,* **23**: 143–146.