STUDIES OF EYE PIGMENTS OF DROSOPHILA.

I. METHODS OF EXTRACTION AND QUANTITATIVE ESTIMATION OF THE PIGMENT COMPONENTS[1]

BORIS EPHRUSSI AND JEAN LANE HEROLD
The Johns Hopkins University, Baltimore, Maryland

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INTRODUCTION

MICROSCOPIC observation, as well as solubility tests and studies of the effects of gene substitutions, have shown that the normal, wild type eye color of *Drosophila melanogaster* is due to the presence of two pigments, red and brown. These two pigments are the end products of two largely independent chains of reactions. Recent work on the genetic control of eye color in Drosophila was almost entirely confined to the reaction chain leading to the formation of brown pigment. The hormone-like diffusible substances derived from tryptophane and representing intermediate links of this chain were the most intensively studied phase of this process. The study of the reaction chain resulting in the formation of red pigment was on the contrary very much neglected and so was the study of the end products of both reaction chains—namely, the red and brown pigments themselves.

Both technical and theoretical factors were responsible for this uneven advance. Technically, the diffusible nature of the hormone-like substances makes them particularly accessible for a very direct experimental attack. The intermediate products involved in the formation of red pigment offer no such technical advantages. The origin of the red pigment, in so far as we know, is entirely intracellular and local. The study of the pigments themselves has encountered technical obstacles, chiefly in finding suitable solvents, which have greatly retarded the understanding of their chemical nature. Only lately a most promising study by BECKER (1939, 1942) somewhat clarified the chemical nature of the pigments, but this lead was broken by BECKER'S death on the Russian front.

Two theoretical considerations may be added to the above technical ones. In the early phases of the work under discussion it was plausible to assume a rather direct relationship between the diffusible substances and the genes controlling their production. It was a reasonable hope that, starting from the tangible diffusible substances, it would be easier to trace back to the genes the whole chain of intermediate reactions, connecting the genes with the characters they control. This possibility had a special appeal to certain investigators, while to others the diffusible substances were of particular interest as means for the discovery of spatial and temporal correlations in development. Whatever the reason in each particular case may have been, the net result is that the chain of reactions leading to the formation of brown pigment is now rather well defined, while that leading to the formation of red is almost entirely un-

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known. The existence of distinct phases in the latter process may be inferred from the effects of some gene substitutions, but their more detailed analysis must await the introduction of new methods. It is the authors' conviction that the study of the pigments, particularly from the quantitative angle, should permit deductions as to the nature of the underlying reactions. It was with this expectation that the work to be reported under the above general title was begun. The purpose of this first report is to describe methods for the quantitative estimation of eye pigments of *Drosophila melanogaster*.

HISTORICAL

As indicated above, several different lines of investigation have provided evidence that the characteristic color of the eye of wild type Drosophila is due to the simultaneous presence of two pigments. First should be mentioned histological observations. These were inaugurated by JOHANNSEN (1924), who described in the primary and secondary pigment cells of the ommatidia the presence of two distinct types of pigment granules, some "purplish-red," others "ochre-yellow." JOHANNSEN also studied several mutant types, among them sepia, purple, vermilion, ruby, pink, etc., and concluded that their eyes differ from those of wild type flies in the amount and distribution of the two pigments.

Descriptions of the pigments in sections of Drosophila eyes, confirming JOHANNSEN'S findings, are given also by CASTEEL (1929), SCHULTZ (1935), and MORI (1937). The latter two workers, in addition to the histological study of the pigment in the imagoes of a variety of mutant types, have given accounts of the pigment deposition in the earlier stages of the development of the flies. It may be added that a study of the cytology of the eye of *Drosophila pseudoobscura* (COCHRANE 1937) revealed in this species a pigment situation strikingly similar to that described by JOHANNSEN in *D. melanogaster*.

Gene substitutions have provided another and, at first, independent line of evidence for the existence of two different pigments in the eye of Drosophila. WRIGHT (1932) was the first to suggest that the total absence of pigment in the double recessive scarlet brown may be explained on the assumption that the mutations at these two loci suppress respectively one of two independent and complementary pigmentation processes. A similar conclusion was independently reached by GLASS (1934), who identified the products of the complementary pigmentation processes of WRIGHT with the red and yellow pigment granules of JOHANNSEN.

The study of Drosophila eye colors by MAINX (1938), who in a single extensive piece of work combined the two above methods of investigation, deserves special mention. While other authors have studied the histological picture of different eye color mutants, MAINX was the first investigator primarily guided in his histological study by a deduction from the results of gene combinations. Curiously enough, the starting point of his work is again the "white" eye of the scarlet-brown combination, and his deduction is identical with that of WRIGHT and GLASS. MAINX has prepared combinations of more than 25 different eye color mutants with scarlet (*st*) and with brown (*bw*)

and has shown that (1) the eye color of any mutant, say *x*, is equal to the sum of the colors of the two double recessive combinations *x st* and *x bw*, and (2) the observation of the combinations of any two eye color mutants with scarlet and with brown, say *x st* and *x bw;* and *y st* and *y bw*, permits the prediction of the color of the combination *x y*. This work, although not really quantitative, has lent the strongest support to the idea of two independent pigment components. The differences between the various mutant races appear to be due primarily to the independent variation in the content of the two pigment components.[2]

On the chemical side we find first an incidental remark in HERTWECK'S (1931) paper concerned with eye morphology. HERTWECK points out that while the red color of the Drosophila pigment suggests its carotinoid nature, its insolubility in acetone, chloroform, and benzene does not support this idea. On the other hand, the solubility of the pigment in HCl indicates that it is not a melanin.

A systematic chemical study of Drosophila eye pigments has been undertaken by SCHULTZ. Unfortunately his results were published only in the form of condensed reports (MORGAN, BRIDGES, and SCHULTZ 1930, 1931, 1932) and in a review (SCHULTZ 1935) devoted to general aspects of gene action. These publications contain no technical details and thus offer no lead for further investigations. From SCHULTZ'S publications, however, we observe that "the red and yellow granules actually correspond to red and yellow pigments which are closely related, but can be separated by means of their solubility differences"; that these pigments have different absorption spectra, which are modified by changes of the pH and by oxidation and reduction (of smears of eyes; reversible oxidation could not be obtained *in vitro*); that they are water soluble, but do not dissolve in any of the usual organic solvents; and that they are dialysable —that is, of small molecular size. It will be seen below that most of these observations are corroborated by later work, but that the solubility in water solutions of the yellow pigment is negligible and that reversible oxidation and reduction of both pigments can be readily observed *in vitro*. The close relationship of the two pigments, according to SCHULTZ, is demonstrated by the fact that they can be converted "by certain treatments" into a third, brown pigment. SCHULTZ'S general conclusion is that the yellow and red pigments are probably "so simply related to each other as oxidation-reduction products."

Next should be mentioned a short note by LAKI (1935–36) who finds that a pigment extracted in water from the eye of wild type Drosophila (presumably the red component) shows the properties of a pH and redox indicator. Much more important information, however, is contained in the above mentioned work of MAINX, who, along with his histological studies, carried out some pigment extractions. MAINX finds in the wild type eye two pigments strikingly different in their solubilities: a red pigment, which is water soluble and a redox indicator, and a brown pigment insoluble in water; the latter is extremely sensitive to alkali, in which it can be dissolved with loss of color. Neither pig-

[2] The changes in pigment content are sometimes accompanied by changes in distribution and quality of the pigment components.

ment is soluble in alcohol, ether, petroleum ether, carbon disulphide, chloroform. These two pigments correspond to the two pigment components assumed on the basis of gene combinations (see above). The eye of the mutant *st*, for instance, contains nothing but the water soluble red pigment; that of the mutant *bw* only the water insoluble brown pigment. *In vivo* both pigments are thought to be bound to a protein carrier.

Finally, reference should be made to two papers by BECKER. The first (1939) deals mainly with the eye pigments of Ephestia (also dependent on diffusible substances), but a few other pigments are also considered, among them those of Drosophila. On the whole, BECKER's results are in agreement with those of MAINX; however, a discrepancy appears as to the solubility of the brown component in acid water solutions. According to BECKER, the brown pigment from finely ground fly heads may be dissolved in HCl-2N. BECKER also reaches the conclusion that there are two distinct pigments in the wild type eye. The red one is very sensitive to acids and rather stable in alkali. The brown behaves in the opposite way. Both are pH and redox indicators. The red pigment (and probably the brown one) are of small molecular size. The brown pigment can be benzoylated, the red cannot. BECKER also is inclined to think that *in vivo* the pigments are bound to a protein carrier; the color changes accompanying the action of denaturing agents are indicative of such a linkage. The eye of Calliphora is found by BECKER to contain one single pigment closely similar to but not identical with the brown pigment of Drosophila. For the pigments of the Drosophila and Calliphora type he proposes the generic name "Ommatins," subdividing them into "Phaeommatins" (brown pigment of Drosophila) and "Erythrommatins" (red component of Drosophila); the red pigment of Drosophila, however, is included in the Ommatin group subject to the confirmation of its close relationship with the Phaeommatins.

In his second paper (1942) BECKER describes the presence of Ommatins in several other groups of insects and reports a more detailed study of their properties. All Ommatins have a common physiological characteristic: their dependence on the tryptophane→kynurenine chain of reactions—that is, their chromophore group is probably derived from kynurenine. They all are different from melanins and carotinoids and show a series of common chemical characteristics. Prominent among these are: small molecular size, linkage with proteins, elementary chemical composition, color and behavior as pH and redox indicators, halochromism, stability in acids, instability in alkali, and their solubilities. They are insoluble in the usual organic solvents, poorly soluble in water, soluble in dilute alkali, very easily soluble in absolute formic acid and especially in acid alcohols. On the basis of this definition, BECKER now excludes the red component of Drosophila from the group of Ommatins.

To this brief and incomplete statement of BECKER'S results we should like to add that, although his aim was not primarily chemical, his work has undoubtedly provided us with the very few known chemical characteristics of the Drosophila eye pigments indicative of their chemical structure, and that in his paper we find the first useful indications concerning the solubility of the brown pigment component.

In none of the studies thus far described is there any attempt to estimate quantitatively the pigments of Drosophila. The interest of developing such a technique, however, has been felt by a few investigators. Their efforts may be said to belong to two different categories, one of which is the attempt to determine the pigment content of individual whole eyes by reflection spectrophotometry. VAN ATTA and VAN ATTA (1931) and later FARDON and CARROLL (1937) have published the results of some measurements of this sort. The analysis of such data, however, especially in quantitative terms, seems very hazardous, particularly in view of the now well established presence of two unevenly distributed pigments. Moreover, it will be shown below that the pigment content is a function of many external factors and therefore subject to important individual variations. For these two reasons it will be difficult to make adequate use of techniques built on similar principles.

The other way to estimate pigment content of course is by extraction followed by some sort of measurement. Prior to the publication of BECKER'S work, the difficulties involved here were mainly in the ignorance of suitable solvents. MONOD and NEEFS in 1938 claimed to have worked out a satisfactory method. Fly heads separated from the bodies were kept for two days in absolute alcohol, then dried, ground, and extracted with water. The extracts were then rapidly boiled, centrifuged, and the pigment concentration determined photometrically. A similar extraction of heads of the mutant white showed that impurities may be disregarded, the absorption of light by these being of the order of one percent of the total absorption by extracts of wild type heads. The data published by MONOD and NEEFS are too few to permit a judgment on the value of the method. We will point out, however, that it disregards the presence of two pigments. From the results of MAINX and BECKER it is clear that the brown component is certainly not extracted by the MONOD and NEEFS procedure.

Early in 1940 DR. G. W. BEADLE kindly communicated to the senior author that DOCTORS E. L. TATUM and C. W. CLANCY had found it possible to extract the red pigment with 30 percent ethyl alcohol acidified with HCl to pH 2.0 and the brown pigment with absolute methyl alcohol containing one percent by volume of dry HCl. Work started by the senior author on the basis of this communication was interrupted by the war. A paper by CLANCY (1942) dealing with the mutant claret contains a description of a technique of pigment measurement making use of the above solvents, as follows. Fly heads (usually 100 or more) immediately after decapitation are placed in a vial containing 2–3 cc of acid ethyl alcohol. After several days the liquid is removed and a fresh supply of solvent added. After a few days more the two extracts are combined, filtered, and pure solvent is added up to a volume of 10 cc. The relative concentration of pigment is measured photometrically at 440 mμ. The extracted heads are rinsed in absolute methyl alcohol and placed for two to three days in acid methyl alcohol for extraction of brown pigment. After reduction with sodium hydrosulfite the measurement of the extract is performed as above. This method of "double extraction" was checked by the extraction of flies homozygous for *st* or *bw*—that is, containing only one or the other pigment component.

The technique used by CLANCY will be discussed in the course of presentation of our own results. As will be seen in the following pages, an investigation of different aspects of the technique based on the use of the solvents mentioned was found to be desirable.

EXPERIMENTAL

We want to emphasize at this point that our aim was to work out a simple and rapid technique permitting extraction and measurement of low pigment concentrations in small volumes of solvent, these conditions being imposed by the nature of the problems to be studied with the help of this technique. We shall first report the results of extraction of the pigment from flies containing only one of the pigments—that is, from *st* or *bw* flies. Then we shall present our experiments on "double extraction" and, finally, the results of our analysis of some causes of variation in pigment content and indicate the precautions which have to be taken to assure the maximum reproducibility of data.

Equipment

All extractions were made in glass-stoppered Pyrex glassware. Depending on the pigment concentration, either 5 and 10 cc cylinders or 100 cc bottles were used.

For the measurement of light absorption, when a sufficient amount of pigment extract (5 cc) was available and measurements at a wide range of wavelengths desired, a Coleman photoelectric spectrophotometer was used. When small volumes of extracts of low concentration were worked with and when consecutive measurements of an undisturbed sample over a period of time were desired, a Pulfrich photometer equipped with 50 mm long microcells (volume approximately 1 cc) and light filters S 43, S 47, S 50, and S 53 (wave length centers of gravity at 430, 450, 500 and 530 mμ) was used.

The results of all the absorption measurements will be given in terms of extinction, E ($E = \log I_0/I$, where I_0 is the incident, and I the transmitted light) this value being proportional to concentration. It will be remembered that E is also proportional to the stratum thickness, L. In the tables E has not been divided by L, since all comparisons are made between measurements obtained with the same instrument.

Unless stated otherwise, all extractions were performed in the dark at 25°C.

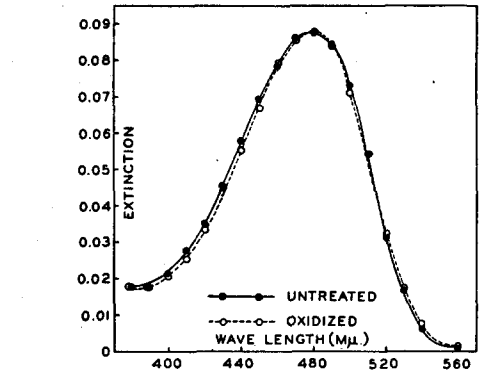
Red pigment component

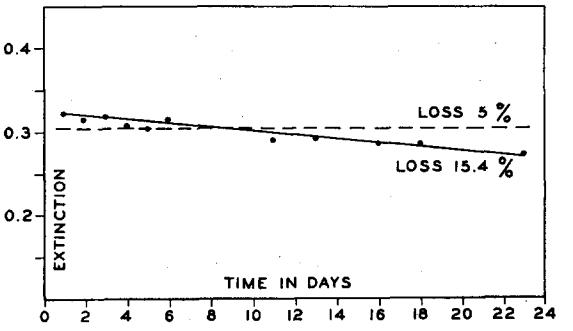
Solvent

As indicated above, the solvent used for the extraction of the red pigment is 30 percent ethyl alcohol acidified to pH = 2.0 (to be referred to below as AEA). This pH is obtained by adding 1 cc of pure concentrated HCl to 1000 cc of alcohol. The pH of freshly prepared solvent was always checked electrometrically, using a glass electrode. Consecutive measurements on samples of the same stock of solvent, extending over several months, confirmed CLANCY'S (1942) statement that the pH of AEA is perfectly stable.

Extraction

When heads of *st* flies, separated from the bodies, are placed in AEA, the solvent soon becomes colored orange yellow, which color gradually increases in intensity. After 48 hours the greater part of the pigment has diffused from the





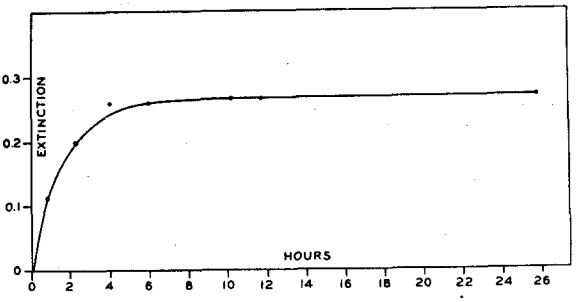


FIGURE 1 (above).—Absorption curves of red pigment extracted in AEA (Coleman).

FIGURE 2 (center).—Loss of absorption of an extract of red pigment on standing (Pulfrich, 470 mμ). The dotted line cuts the curve at the point of a 5 percent "loss."

FIGURE 3 (below).—Extraction of red pigment in AEA followed photometrically (Pulfrich, 470 mμ).

heads into the solution. The absorption curve of such a solution is shown in figure 1. It may be seen that the light absorption is maximum at 480 mμ and decreases rapidly on both sides of this maximum.

Observation of the heads after a 48 hours' extraction shows that most eyes are completely extracted and appear nearly white, while a few still retain variable amounts of pigment, their color ranging from yellow to dark red. Although, according to CLANCY, AEA extracts the red pigment "readily and completely," the complete extraction under the described conditions obviously requires a much longer time. In fact, examination of CLANCY's data shows that in his experiments the extraction time varied from 12 to 38 days, and our own experience indicates that often some unextracted heads can be found even after a week or ten days of extraction.

This very long extraction time is not only inconvenient, but also introduces an error, because, as CLANCY has already noticed, the solutions of red pigment are not quite stable. The "loss" of pigment can be followed photometrically. The results of such an experiment are shown in figure 2 where the extinction (a value directly proportional to the concentration of pigment) is plotted against time. In this experiment an extract was filtered and kept at 25°C. The absorption was measured on samples of the extract at various time intervals. It may be seen that the gradual decrease of absorption corresponds to a loss of 5 percent of pigment concentration per week. Thus a long extraction time introduces an appreciable error through pigment destruction.

The variability in the rate of extraction of individual heads suggested that its cause is probably mechanical and could be remedied by mechanical means. A comparison was made of the rate of extraction from (a) entire heads and (b) from heads split longitudinally (between the eyes) with the help of a chip of a safety razor blade. After 24 hours the eyes of the latter sample were nearly white,[3] while those of the former remained strongly colored. Determination of the exact time required for the complete extraction of halved heads was made by following the light absorption of an extract. A sample of heads was placed in a large amount of AEA, and the absorption of samples of the extract was measured at intervals. Figure 3 shows that the extraction is complete within 10 hours.

pH

As pointed out by several authors (see above), the extracted red pigment shows the behavior of a pH indicator. If a few drops of dilute NaOH are added to an extract prepared in the described manner, its color turns red. Although the pH of the solvent has been found to be extremely stable and fluctuations of acidity of a stock solution may be discounted, the question was raised of how far the results of measurements would be affected by small differences in the pH of solvents prepared at different times. Several samples of an extract of *st* pigment were diluted with equal amounts of 30 percent ethyl alcohol acidified to various pH values. The final pH of each of the mixtures as well as the light absorption was measured. Figure 4 shows that the extinction, measured at 470 mμ (that is, in the vicinity of the absorption maximum) is independent of acidity variations between pH 1.5 and 2.5.

[3] Longer extraction of the halved heads does not remove the remaining very pale purplish color of the eyes which will be shown later to be due to a small amount of brown pigment.

In another experiment of the same type the light absorption of different samples of the same extracts adjusted to four different pH values was measured at four different wave lengths. Figure 5 shows a marked shift of the absorption maximum towards longer wave lengths at higher pH values. The change, cor-

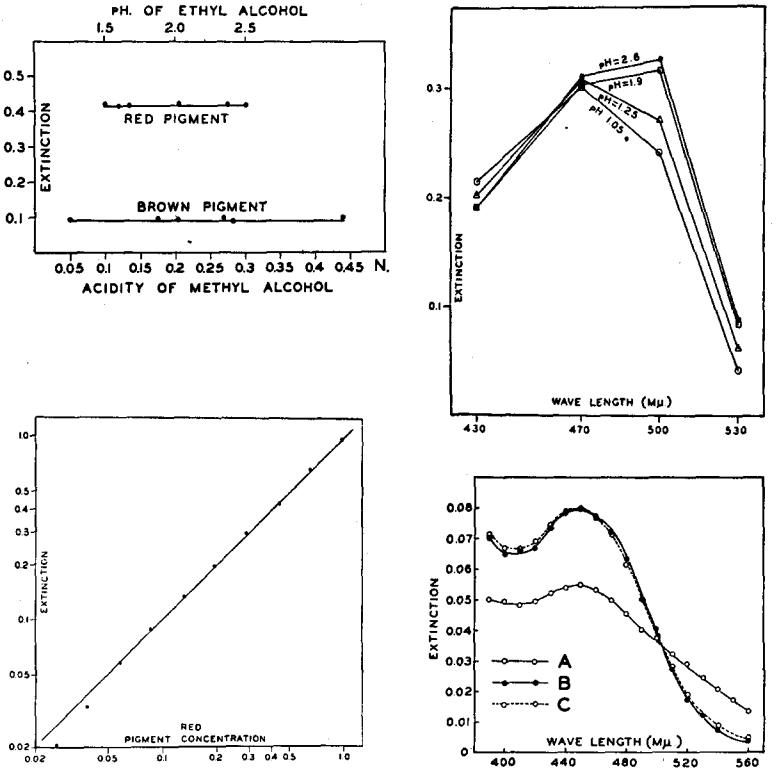


FIGURE 4 (upper left).—Absorption, at 470 mμ, of red and brown pigments in solvents of different acidities (Pulfrich). The curve for red pigment is referred to the upper abscissa, that for brown to the lower.

FIGURE 5 (upper right).—Effect of pH on the absorption curves of red pigment (Pulfrich).

FIGURE 6 (lower left).—Relation between concentration of red pigment and extinction (Pulfrich, 470 mμ).

FIGURE 7 (lower right).—Absorption curve of brown pigment extracted in AMA. A—untreated extract, B—oxidized, C—oxidized five days later (Coleman).

responding of course to the visible color change, is particularly obvious at 500 mμ, while no significant change is seen at 470 mμ.

Oxidation and reduction

The experiments here reported confirm earlier observations that the red pigment acts as a redox indicator. If a few crystals of sodium hydrosulfite are added to an extract of red pigment, the orange color of the latter immediately disappears, and the solution becomes colorless. This reduction is reversible: on standing in the presence of air, the solution gradually recovers its original

color. Therefore, the problem arose as to whether or not the extracts prepared at different times contain pigment in the same state of oxidation. Experiments were performed in which the light absorptions of two samples of the same extract, one untreated, the other oxidized by addition of a drop of 3 percent by volume of H_2O_2, were measured. Figure 1, representing the results of one of several concordant experiments, clearly shows that the pigment extracted in the described way is in the state of maximum oxidation. (It should be noted that this permits no conclusion as to the state of oxidation of the pigment *in vivo*.) No extra precaution is therefore needed to control the oxidation state of the extracts.

Applicability of the method to determination of concentration

Methods of photometric determination of concentration rest on the validity of Beer's law, according to which there is a linear relationship between extinction and concentration. It was necessary therefore to ascertain that solutions of red pigment obey Beer's law. A concentrated sample of red pigment was prepared. The extinctions of this solution and of several successive dilutions were measured with the Pulfrich photometer at 470 mμ. The result of the experiment is given in figure 6, where extinction is plotted against relative concentration. The points are experimental, the straight line theoretical. It is clear that the red pigment obeys Beer's law within the concentration range corresponding to extinctions from 0.05 to 1.0 under the conditions of the measurements. In terms of heads of *st* flies this corresponds approximately to from 12 to 250 heads per 100 cc of solvent.

In determining the number of heads and the amount of AEA to be used for a single measurement, two additional factors must be considered. First, in the case of the Pulfrich photometer the maximum accuracy of readings is in the neighborhood of 70 percent transmission which corresponds to an extinction of 0.155. Such a concentration is given, under the conditions of culture described below, by approximately 40 heads in 100 cc of AEA. Second, there are naturally individual variations between flies, so that a greater accuracy will be reached with extracts from numerous heads. Our experience shows that samples of 30–40 heads from the same culture bottle give values rarely differing by more than 2 percent.

Impurities

The extracts discussed in the preceding pages are quite transparent. When the heads are split to permit a faster extraction, small particles (mainly of brain tissue) may eventually be found suspended in the solution. But so long as *st* flies are dealt with—that is, so long as strongly pigmented eyes are extracted—only a few heads per 1 cc of solvent are required to produce a pigment concentration adequate for photometric measurements. At this concentration the turbidity is so low that it may be disregarded. When numerous heads have to be extracted in a small volume of solvent, the turbidity due to small fragments of tissues may be eliminated by filtration under reduced pressure through a sintered glass filter.

It is clear that AEA also extracts absorbing substances other than the pigment. To what extent these impurities affect the measurements of pigment concentration may be tested by extracting heads of flies the eyes of which contain no visible pigment. Three such experiments were performed using white (*w*) and white scarlet flies. The results are given in table 1 where the extinction (E) is calculated per ten heads in 1 cc of AEA. The comparison of the values obtained in these experiments with data for *st* (table 3, to be discussed later) shows that the impurities correspond to less than 0.3 percent of the total absorption of scarlet extracts. It is clear also that in working with less colored mutants, where impurities have a greater importance, appropriate corrections may easily be made.

TABLE 1

Photometric determinations (Pulfrich, 470 mμ) of absorption by impurities extracted in AEA and AMA. E—extinction per 10 heads in 1 cc of solvent. N—number of heads.

EXP. NO.	N	GENOTYPE AND SEX	SOLVENT	E	AVERAGE
B-57	335	*w* ♂	AEA	0.012	
VII-72	300	*w; st* ♂	AEA	0.010	0.010
VII-80	350	*w; st* ♂	AEA	0.009	
B-57	362	*w* ♀	AEA	0.013	
VII-72	300	*w; st* ♀	AEA	0.015	0.014
VII-80	300	*w: st* ♀	AEA	0.015	
B-33	145	*w* ♂	AMA	0.005	0.005
B-37	373	*w* ♂	AMA	0.004	
B-33	179	*w* ♀	AMA	0.007	0.006
B-37	400	*w* ♀	AMA	0.005	

Brown pigment component

Solvent

The acid methyl alcohol (henceforth to be referred to as AMA) used for the extraction of brown pigment is prepared by bubbling HCl gas through absolute methyl alcohol. The desired acidity of 1.0 percent by volume (approximately 0.270 N) is determined by titration with decinormal NaOH. The solvent is not stable and loses its acidity on standing.

Extraction and properties of brown pigment

Entire heads of *bw* flies, placed in AMA, lose their pigment rapidly; after 24 hours the eyes are white. The extract is of a brownish color. It becomes yellow on addition of H_2O_2 and pink on reduction with sodium hydrosulfite. The color of the oxidized solution varies with changes of the pH. In more acid solutions it becomes pinkish.

In a fresh extract the pigment is obviously in a state of partial oxidation, and a precise study of the extracts must begin with its complete reduction or oxidation. Between these two possibilities oxidation was chosen because it was observed in preliminary experiments that reduction is frequently accompanied by appearance of turbidity.

The absorption curve of an untreated, freshly prepared brown pigment extract in AMA is represented in figure 7 (curve A). Curve B shows the absorption of the same solution after it has been oxidized under the conditions to be described in the next section. It may be seen that both curves have a peak at 450 mμ and probably a second peak in the near ultra-violet and that the absorption in the major part of the range and especially at the peak is increased by oxidation.

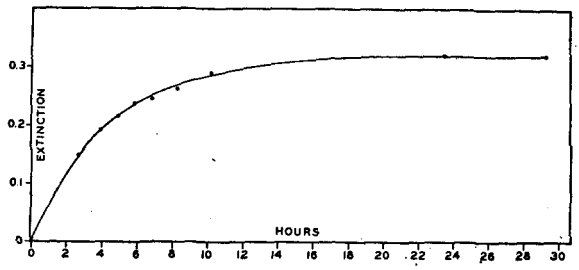
Curve C represents the absorption of a fraction of the same extract kept five days at room temperature and oxidized at the end of this period. Its comparison with curve B shows that the extract is perfectly stable at room temperature.

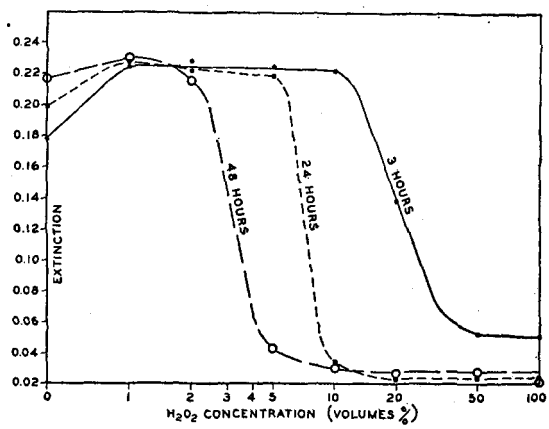
The time required for complete extraction of *bw* flies may be determined by the procedure already used for the red pigment. Figure 8 shows that the extraction is complete in less than 24 hours.

The extracts of brown pigment contain no gross turbidity. They may be used for photometric measurements directly. The amount of extracted impurities has been determined by extracting heads of white flies. The extracts were oxidized and their absorption measured at 470 mμ. The data recorded in table 1 compared to those in table 4 (to be discussed later) show that the impurities are responsible for approximately one percent of the total absorption of the extract of *bw* heads. The reservations made in connection with the impurities contained in red pigment solutions are equally valid here.

Oxidation

The precautions required for the oxidation of brown pigment may be illustrated by the following experiment. A fresh solution of brown pigment is divided into eight samples of 5 cc each. To each of the samples one drop of hydrogen peroxide of a known and different concentration is added. The control receives one drop of distilled water. After three hours the absorptions of the different samples are measured at 470 mμ. Figure 9 shows that the samples oxidized with H_2O_2 of 1.0, 2.0, 5.0, 10.0 percent by volume show identical extinctions, all higher than that of the control, and that the samples oxidized with higher concentrations of H_2O_2 show a decrease of absorption. In other words, drastic oxidation destroys the pigment. The destruction is faster in stronger H_2O_2 solutions. The measurements repeated after 24 and 48 hours (see figure) show a similar destruction in samples to which lower concentrations of H_2O_2 were added. The samples oxidized with 1.0 and 2.0 vol. percent H_2O_2 are stable for 48 hours. This experiment indicates the concentration of H_2O_2 to be used. It will be noticed also that the absorption of light by the control has markedly increased in the intervals between measurements, obviously through oxidation by the oxygen of the air; this serves only to emphasize the necessity for complete oxidation before measurements are undertaken.





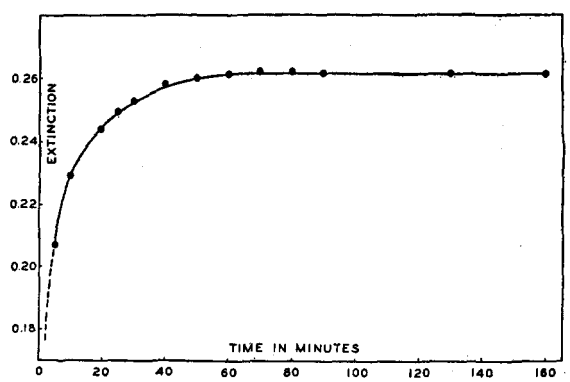
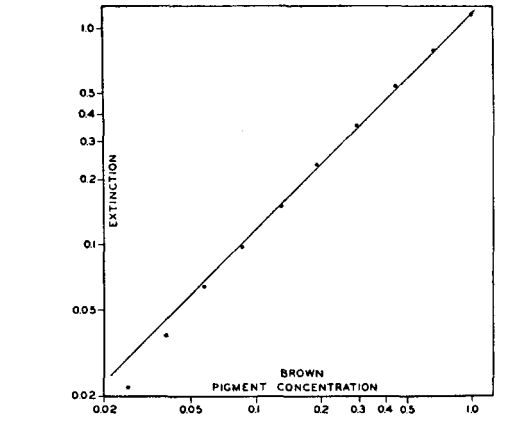


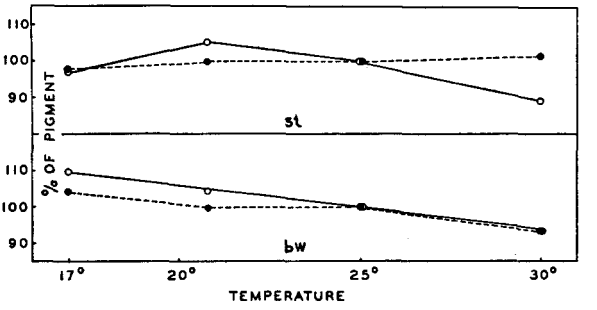
FIGURE 8 (above).—Extraction of brown pigment in AMA followed photometrically (Pulfrich, 470 mμ).

FIGURE 9 (center).—Absorption of brown pigment extracts oxidized with various concentrations of H_2O_2 (Pulfrich, 470 mμ).

FIGURE 10 (below).—Oxidation of brown pigment followed photometrically (Pulfrich, 470 mμ).

In our experiments one drop of 2.0 percent by volume of hydrogen peroxide per 5 cc of extract was used for the oxidation of the brown pigment. The time to be allowed for full oxidation, at 25°, was determined by following the color change photometrically. The results of one of these experiments are given in figure 10 where it may be seen that the extinction has reached a stable value





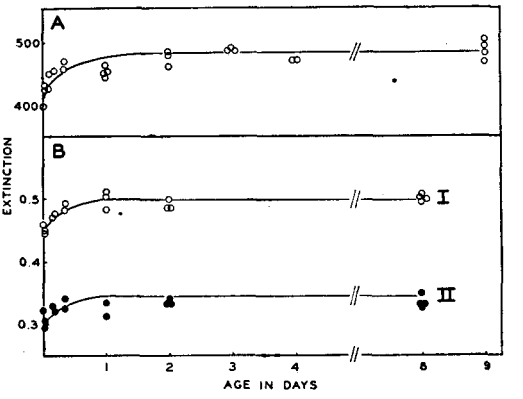


FIGURE 11 (above).—Relation between concentration of brown pigment and extinction (Pulfrich, 470 mμ).

FIGURE 12 (center).—Effect of temperature on the pigment content in *bw* and *st* ♂♂ (Pulfrich, 470 mμ). E calculated per 10 heads in one cc of solvent. Details in text.

FIGURE 13 (below).—Change in content of brown pigment with the age of *bw* ♂♂. Figures A and B represent the results of two different experiments. Curves A and B-I give the extinctions of oxidized extracts, curve B-II those of untreated extracts. E calculated per 10 heads in 1 cc of AMA (Pulfrich, 470 mμ).

70 minutes after addition of H_2O_2. Our measurements were usually made one and a half to two hours after the addition of hydrogen peroxide.

Determination of relative concentration

The applicability of Beer's law was tested for the brown pigment by a procedure similar to that employed with the red pigment. All tested samples were oxidized as described in the preceding paragraph. Figure 11 shows that the brown pigment follows Beer's law within the concentration range corresponding to extinctions from 0.06 to 1.0 as measured by the Pulfrich photometer. This corresponds to from 12 to 200 heads of *bw* flies grown under our standard culture conditions (see below) in 10 cc of solvent. The number of heads for optimum photometric determinations was found to be of the order of 40 heads per 10 cc of AMA.

Double extraction

Now that the behavior of the brown and red pigments in the solvents used for their extraction is known, the question may be raised whether these same solvents may be used for a "double extraction"—that is, for separation of the two pigments from eyes containing both (wild type eyes for example). First to be ascertained in this connection is the behavior of each of the pigments with respect to the solvent so far used for the extraction of the other.

If *st* heads are placed in AMA, it is observed that this solvent extracts the red pigment very easily and completely. A double extraction, beginning with the extraction of the brown component in AMA is therefore impossible. On the other hand, if *bw* heads are placed in AEA for a period of time sufficient for the extraction of red pigment, the solvent remains colorless to the naked eye. It seems possible therefore to extract, from a wild type head for example, first the red pigment with AEA and to follow this extraction by an extraction with AMA to remove the remaining brown pigment.

On closer examination, however, difficulties appear which have already been noticed by CLANCY (1942), who, as stated above, used double extraction with the reservation that the method is open to criticism. In the first place, the solvent used for the extraction of red pigment (AEA) seems to render insoluble a part of the brown pigment. Thus *bw* eyes, placed first in AEA for 24 hours, then extracted 24 hours in AMA, retain a slight but distinct purplish tinge, while control eyes, extracted in AMA directly, become white. Second, AEA does extract some brown pigment. How serious these two circumstances are may be tested by experiments in which equal samples of *bw* heads are extracted either directly in AMA or after pretreatment with AEA. Five such experiments were performed, and the photometric determinations of pigment concentration consistently indicated loss of pigment which, in the different experiments, amounted to 25.6, 26.2, 27.0, 29.6, and 30.6 percent (for details compare columns B, C, and D, table 2). These losses are much greater than assumed by CLANCY. He measured the absorption of the extract of *bw* heads in AEA after 37 and 56 days and compared the obtained values with those given by extracts of *bw* heads in AMA. The loss was found to be 15 percent. There are reasons, however, to suspect that the use of two different solvents invalidates his conclusion. It is possible, moreover, that in CLANCY'S experiments part of the brown pigment was destroyed due to long standing in AEA.

TABLE 2

Comparison of the amounts of brown pigment obtained by double extraction of wild type and by double and direct extraction of brown flies. Wild type and brown flies were grown together in the same bottles. In each experiment the three extractions were practiced on identical numbers of heads of flies of the same sex in the same volume of solvent. E—extinction of a sample at 470 mμ; M—average of samples of the same experiment.*

EXP. NO. AND INSTRUMENT USED	A WILD TYPE DOUBLE EXTRACTION		B BROWN DOUBLE EXTRACTION		C BROWN DIRECT EXTRACTION		D LOSS OF BROWN IN DOUBLE EXTRACTION %	E B IN % OF A	F C IN % OF A
	E	M	E	M	E	M			
IV-85 Pulfrich	1.369 1.410	1.390	0.985 0.973	0.979	1.298 1.358	1.328	26.2	70.5	95.5
VII-88 Coleman	—	—	0.283 0.283	0.283	0.410 0.405	0.408	30.6	—	—
IX-59 Coleman	0.926	—	0.610	—	0.867	—	29.6	66.0	93.7
IX-63 Pulfrich	1.180	—	0.808	—	1.108	—	27.0	68.5	92.8
B-29 Pulfrich	—	—	0.821	—	1.105	—	25.6	—	—
							m=27.8	m=68.3	m=94.0

* Not corrected for number of heads and volume of solvent.

What parts are played by the solubility in AEA and by the fixation of a certain fraction to AMA in the loss of brown pigment during double extraction is not known, but we believe that the greater part of the loss is due to the first factor.

These experiments, performed on *bw* eyes, do not necessarily mean that the same situation obtains in the extraction of wild type eyes. Certain differences in the behavior of pigments in these two types do exist. For instance, wild type eyes retain much less color after double extraction than *bw* eyes; however, there is no evidence that the brown pigment from wild type eyes is less soluble in AEA than that from *bw* eyes, and so long as this is not proved, the method of double extraction as attempted here and used by CLANCY must be considered inadequate for the estimation of brown pigment.

There still remains the possibility (which could be of real advantage in certain cases) that the measurement of the red pigment extracted with AEA from eyes containing both pigments might not be disturbed by the admixture of some brown pigment, the light absorption of the brown pigment being so much smaller than that of the red pigment. Whether or not this is so has been tested by experiments in which extracts in AEA were prepared from (a) a

certain number of *st* heads and (b) an equal number of *st* heads plus the same number of *bw* heads. The extinctions of two such samples were found to differ by less than one percent. It is clear therefore that the red pigment may be estimated in extracts "contaminated" by some brown pigment, provided the proportion of red is far in excess of brown pigment.

Numerous other attempts at double extraction will not be described. It will only be pointed out that theoretically determination of the respective concentrations of two pigments in a mixture is possible when the absorption spectrum of each is known, when one differs sufficiently from the other, and when there is no interaction between them. In such cases the absorption values of the mixture at two adequately selected wave lengths permit the calculation of the concentration of each of the components. However, such determinations are always delicate, and our tests of this procedure, by measuring the absorption of mixtures of brown and red pigment solutions of known concentrations in AMA, have yielded unsatisfactory results.

In view of these considerations, use of the double extraction was restricted to a few special cases and we relied chiefly on separate extractions of the two pigment components, from flies having only one of them at a time. The validity of this method will be discussed after some factors of variation of the pigment content in flies of a given genotype have been considered.

Factors of variation of pigment content

Preliminary determinations of the content of brown and red pigments in the eyes of the mutants *bw* and *st* of our stock raised without special precautions under the usual conditions of Drosophila work have shown great variations in the values obtained. Within the same stocks, determinations made at different times may give values differing by more than 50 percent. Variations of such magnitude would overshadow differences due to genotype, in a comparative study of different mutants for example. Since it is known that external factors influence many characteristics of the flies, including their size, standardization of the culture conditions was attempted. Moreover, the eye color in Drosophila changes with the age of the flies; the causes of the age-change are unknown, and it was thought that eventually it may be responsible for a part of the variations encountered. Pigment content, as a function of age, was therefore investigated in some detail.

Culture conditions

The procedure adopted for this investigation is as follows.

Flies from the stock are fed for one to three days on ordinary Drosophila food "painted" with a heavy suspension of Fleischmann's yeast. Thereafter the flies are transferred into large cotton stoppered glass jars (30 to 50 females per jar) and allowed to lay eggs on yeasted food contained in small metal boxes. After a 20-hour laying period the first larvae usually begin to hatch and may be easily eliminated. The boxes are then placed in petri dishes and the eggs allowed to hatch during a definite (usually four-hour) period. The larvae hatched during this time interval are collected and transferred onto the food on which they will complete their development. This food is prepared according to formula B of BRIDGES and DARBY (1933), reinforced by addition of one per-

cent of Fleischmann's dry powdered brewer's yeast. No Moldex is used. The food (approximately 80 cc per bottle) is slanted and "painted" with a fresh yeast suspension immediately prior to the transfer of larvae. Preliminary experiments have shown that the weight of flies is not affected by the number of larvae grown per bottle, as long as that number is below 175. In our experiments 100 to 120 larvae were raised in one bottle.

These operations are performed at 25°C in accurately controlled incubators. The importance of temperature as a factor of variation in pigment content was shown by experiments of which figure 12 (solid lines) gives an example. In this experiment *st* and *bw* larvae were raised at 25°C up to the moment of pupation and thereafter transferred to four different temperatures (17°, 21°, 25°, and 30°C). The hatched flies were aged (see below) before decapitation. Figure 12 shows clearly that the amount of both red and brown pigments undergo considerable and independent variations with temperature.

Age change

In several experiments *bw* and *st* flies, raised in large numbers under the above described conditions, were isolated within the hour after emergence. At definite times thereafter they were decapitated, the heads extracted, and the absorption of the extracts determined. The results of some of these experiments are represented in figures 13 to 17. Figure 13A shows for the mutant *bw* that the absorption increases during the first 14 hours, at which time the curve reaches a plateau. Figure 13B, curve I, shows a similar result of another experiment of the same type. It is a control to curve II (same figure) which represents the absorptions of the untreated extracts. This curve is exactly parallel to the curve for oxidized extracts (control). Curves A and B of figure 14 give again the absorptions of extracts of *bw* eyes from flies of different ages: flies of series A (curve A) are controls grown under standard conditions, those of series B (curve B) have been raised under conditions of crowding and undernutrition (150 larvae per vial containing 10 g. of food of the same formula without dry yeast). Here the size of the flies was much smaller, the absorption per head much lower, but the general shape of the curve is again similar to that of the control.

Figure 15 shows the absorption curves of extracts from heads of freshly hatched and ten day old *bw* flies. Here the ordinates are given as log E in order to make the shape of the curves independent of concentration differences. The shapes of the two curves are strictly similar; the pigments extracted from young and old flies are identical, and the increase in absorption must therefore be attributed to change in pigment content.

Figure 16 shows the results of experiments similar to the above performed on *st* and vermilion (*v*) flies. Here again we see an increase in absorption with age: this increase is even considerably more pronounced than in *bw* and extends over a much longer period of time; the absorption values are stabilized only four to five days after emergence of the flies. Measurements at different wave lengths of extracts of *st* eyes (fig. 17) show that there is little if any change in the quality of the red pigment either, further indicating that the change with age involves an increase in pigment content.

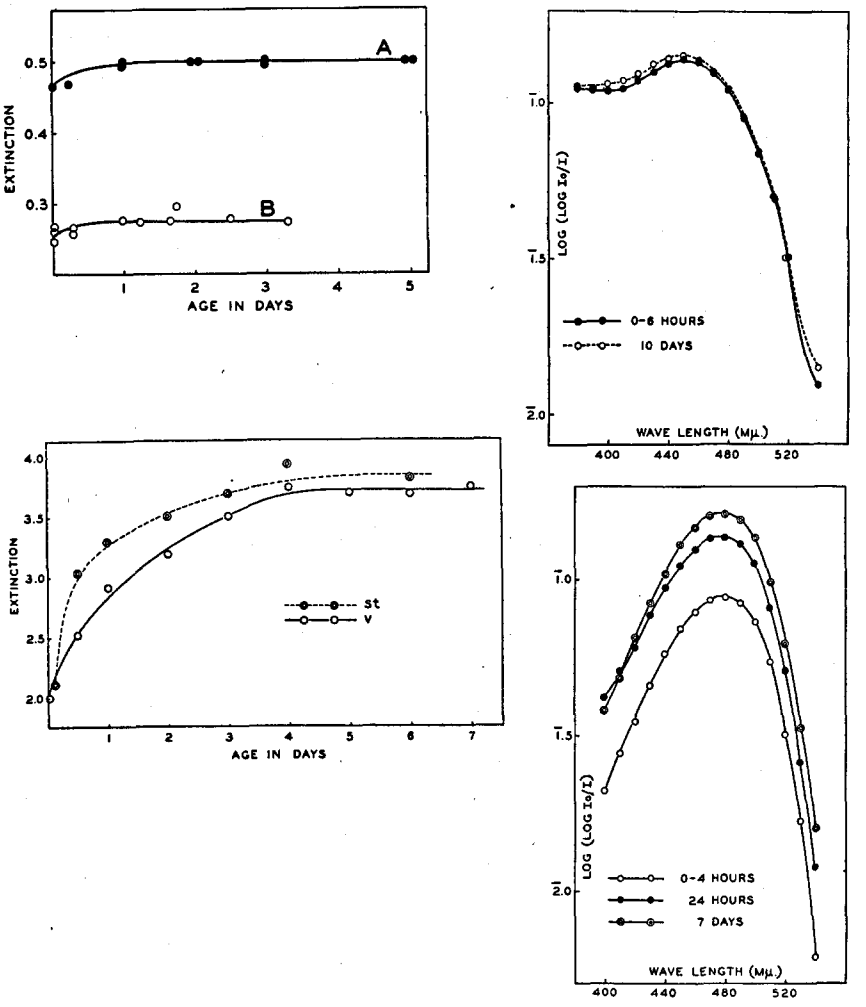


FIGURE 14 (upper left).—Change in content of brown pigment with the age of *bw* ♂♂. A—flies grown under standard conditions, B—flies grown under conditions of crowding (Pulfrich, 470 mμ).

FIGURE 15 (upper right).—Absorption curves of brown pigment extracted from *bw* ♀♀ of different ages. E calculated per 10 heads in 1 cc of AMA (Coleman).

FIGURE 16 (lower left).—Change in content of red pigment with the age of *st* and *v* ♂♂. E calculated per 10 heads in 1 cc of AEA (Pulfrich, 470 mμ).

FIGURE 17 (lower right).—Absorption curves of red pigment extracted from *st* ♀♀ of different ages. E calculated per 10 heads in 1 cc of AEA (Coleman).

The practical conclusion from these experiments is that quantitative pigment measurements must be made on flies which have reached the age of stable pigment concentration. In the experiments reported here, unless otherwise stated, *bw* flies used for extraction were aged for at least two days, *st* and *v* flies for at least five days after emergence.

Analysis of data

The methods of culture, extraction, and measurement described in the preceding paragraphs, applied to the mutants *bw* and *st*, have furnished the data contained in tables 3 and 4. Inspection of these shows that, in spite of the standardized conditions, there remains a considerable variability in the values obtained. In column "E" (extinction of extracts of individual samples of flies) the greatest differences between the highest and lowest values, in percentage of the lowest, are as great as 15.8 for *st* ♂, 7.8 for *st* ♀, 15.1 for *bw* ♂, and 17.9 for *bw* ♀. In the data contained in the column "Average E" (average of the values given by several samples in a single experiment) the variations are slightly smaller. Here the greatest differences (in the above order) are 14.5, 7.8, 13.8, and 15.3 percent. It will be observed that these differences are considerably greater than the differences observed between the different samples within any particular experiment, recorded in the third and eighth columns of tables 3 and 4. This fact shows that the greater part of the variation is due not to the methods of measurement, but to variations in the conditions of development of the flies from experiment to experiment. Several possible reasons have been considered.

1. It was thought that the condition (age, nutrition, size) of the females used as progenitors could have an effect on the pigment content of the progeny. An experiment was performed in which the content of red pigment was compared in the offspring of (a) young *st* females, raised under standard conditions and of (b) old, undersized *st* females from crowded stock bottles. The results, recorded in table 3 (experiments V-99 and V-102) clearly show that the age and condition of the females do not detectably influence the pigment content of the progeny. The differences between the two kinds of offspring are 0.3 percent for the males and 2.1 percent for the females; these are not greater than the usual differences between different samples of a single experiment. These data are in accord with the finding of WARREN (1924) that the size of eggs in Drosophila is independent of external conditions and the size and age of the females.

2. Although the food used in our experiments was always prepared according to the same formula, it was thought that media prepared on different days might be different, due to slight variations in cooking time or to differences in the yeast or in the contaminating bacterial flora. The sections at the bottom of tables 3 and 4 contain experiments in which females were allowed to lay for several days; larvae hatched on successive days were raised on media freshly prepared on each of these days. It may be seen that the differences between the values obtained here are greater than the usual differences between different samples of single experiments, recorded in the upper sections of the same tables. From these results it may be concluded that to test the effects of gene substitutions *ceteris paribus*, different types of flies must be raised simultaneously on the same media. In the experiments described in the next section the *st*, *bw*, and wild type flies were actually raised in the same culture bottles.

3. Comparison of the data in the columns "Weight of ♂♂" shows that the average weight also varies considerably from experiment to experiment, and

TABLE 3

Photometric determinations (Pulfrich, 470 mμ) of red pigment in the mutant scarlet. E—extinction per 10 heads in 1 cc AEA.

	♂♂					♀♀				
EXP. NO.	E	DIFFERENCE %	AVERAGE E	WEIGHT OF ♂♂ IN MGS.	CORRECTED AVERAGE E	E	DIFFERENCE %	AVERAGE E	CORRECTED AVERAGE E	
II-124	4.072 4.064 4.024 4.032	1.2	4.048	0.977	4.143	—	—	—	—	
III-46	3.724 3.776	1.4	3.750	0.997	3.761	—	—	—	—	
III-153	3.688 3.664 3.612	2.1	3.655	0.945	3.868	—	—	—	—	
III-120	3.940 3.956 3.960	0.5	3.952	1.052	3.757	—	—	—	—	
III-176	3.680 3.704 3.632	2.0	3.672	0.958	3.833	—	—	—	—	
III-91	4.012 3.960	1.3	3.986	1.001	3.982	—	—	—	—	
III-9	3.824 3.808	0.4	3.816	1.060	3.600	—	—	—	—	
IV-77	4.132 4.103 4.076	1.4	4.104	1.018	4.031	4.632 4.616 4.676	1.3	4.641	4.559	
V-99	4.012 4.024	0.3	4.018	0.927	4.334	4.684 4.676	0.2	4.680	5.049	Old stock females
V-102	4.004 4.008	0.1	4.006	0.951	4.212	4.584	—	4.584	4.820	Females from young standard culture
V-115	4.012	3.3	4.012	0.935	4.291	4.832	1.8	4.832	5.168	Same females; food prepared on two consecutive days
V-130	4.144		4.144	0.928	4.466	4.748		4.748	5.116	
IV-172	4.068	4.5	4.068	1.052	3.867	4.484	3.6	4.484	4.262	Same females; food prepared on four consecutive days
V-2	4.004		4.004	0.984	4.069	4.572		4.572	4.646	
V-6	4.184		4.184	0.946	4.423	4.644		4.644	4.909	
V-14	4.048		4.048	0.972	4.165	4.528		4.528	4.658	
	m=3.936		m=3.967		m=4.050	m=4.640		m=4.635	m=4.799	

the question was raised as to whether or not the pigment content should be corrected for these weight differences. In tables 3 and 4 the columns "Corrected average E" contain the data resulting from adjusting the average extinction to 1 mg of weight.[4] The differences between the lowest and highest values in

[4] The values for females are corrected by the weight of male sibs.

TABLE 4

Photometric determination (Pulfrich, 470 mμ) of brown pigment in the mutant brown. E—extinction per 10 heads in 1 cc AMA.

	♂♂					♀♀				
EXP. NO.	E	DIFFERENCE %	AVERAGE E	WEIGHT OF ♂♂ IN MGS.	CORRECTED AVERAGE E	E	DIFFERENCE %	AVERAGE E	CORRECTED AVERAGE E	
I-3	0.523 0.509 0.513 0.514	2.8	0.515	0.968	0.532	0.637 0.596 0.621 0.620	6.9	0.619	0.639	
III-3	0.532 0.547 0.547	2.8	0.542	0.954	0.568	—	—	—	—	
I-106	0.498 0.500 0.501 0.501 0.497 0.501 0.500 0.500	0.8	0.500	0.969	0.516	—	—	—	—	
III-76	0.565 0.572 0.570 0.567	1.2	0.569	0.934	0.609	—	—	—	—	
IV-88	0.544 0.544	0.0	0.544	1.035	0.526	0.649 0.679	4.6	0.664	0.642	
IV-115	0.529		0.529	0.981	0.539	0.576		0.576	0.587	Same females; food prepared on three consecutive days
IV-127	0.531	6.2	0.531	1.000	0.531	0.626	11.3	0.626	0.626	
IV-139	0.562		0.562	0.987	0.569	0.641		0.641	0.649	
	m=0.528		m=0.537		m=0.549	m=0.627		m=0.625	m=0.629	

these columns are: *st* ♂: 24.1, *st* ♀: 21.3, *bw* ♂: 18.0, and *bw* ♀: 10.6. The correction obviously does not cancel the differences between the values obtained in the different experiments; as a matter of fact, the differences are greater after correction. A paper by KALMUS (1943), published after the reported experiments were completed, shows that, according to environmental conditions the weight of an individual fly may undergo extensive variation in the course of its life. It is not surprising, therefore, that a correction by weight as applied here does not prove adequate. The correction of the general average of pigment concentration by the general average weight may have a better justification when there is a systematic and considerable difference in weight, due to genotype, for example. Even then, great caution is called for, since it is easily shown that there often is no simple relationship between weight and pigment content. For example, in figure 12, which represents the pigment content as a function of temperature, the dotted lines give the pigment content corrected by weight. It will be seen that, while the red pigment so adjusted became almost independent of temperature, this does not seem to hold for the brown pigment. The

data of figure 14 show that the pigment content of *bw* flies raised under conditions of crowding is approximately 55 per cent of that of control flies. The difference in weight, however, is much less (0.651 mg and 0.969 mg, respectively) and, after correction by weight, the value for pigment content in the flies from the crowded cultures still is only about 80 per cent of the control. Further data pointing to similar conclusions will be found in table 5, to be discussed in the next section.

Validity of the method of separate extractions

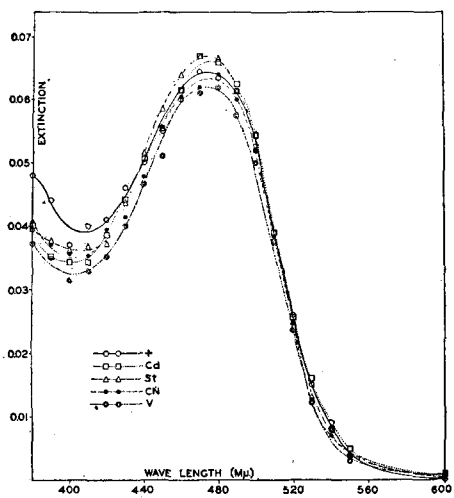
As stated above, the unsatisfactory results of "double" extractions have led us to prefer in our work the separate extraction of the two pigment components from flies in which the formation of one of the components is suppressed by a mutant gene. This second method is obviously based on MAINX's (1938) statement that the total pigmentation of a given mutant, *x*, is equal to the sum of pigments contained in the two double-recessives, *x st* and *x bw*. However, MAINX's statement is a deduction from purely visual observation. Whether or not it holds when translated into strict quantitative terms is evidently an open question which has to be solved in each individual case because there may or may not be interaction between the main gene studied and the genes introduced to eliminate one of the pigments. Here we will limit the discussion to the case of the wild type eye.

First, are the two pigments contained in the wild type eye qualitatively identical with those separately extracted from the mutants *st* and *bw*? Since it has been shown that the determination of red pigment extracted from *st* with AEA is not measurably affected by contamination with small amounts of brown pigment, we have compared AEA extracts of wild type eyes with similar extracts of *st* eyes. Figure 18 shows that the absorption curves of the two extracts are identical. The same figure shows absorption curves of extracts of three other mutants: vermilion (*v*), cinnabar (*cn*) and cardinal (*cd*), all three belonging, together with *st*, to the so-called vermilion group of mutants. Their phenotypes resemble very closely the phenotype of *st:* their eyes contain very little or no brown pigment (SCHULTZ 1935; MAINX 1938). The figure shows that the red pigment extracted from these mutants is similar to that contained in wild type and *st* eyes. Observation of heads after extraction with AEA shows completely white eyes in *v* and *cn*, a slight purplish tinge in *st*, and a considerably darker purplish color in *cd*. This color is exactly similar to that of *bw* eyes after double extraction and is undoubtedly due to the presence of a small amount of brown pigment. From the qualitative point of view, then, the genes *v*, *cn*, and *st* may all be used for the suppression of brown pigment. The greater amount of brown pigment in *cd* calls for caution.

There seems to be identity also between the brown pigments contained in wild type and in *bw* eyes. The three curves of figure 19 represent the absorptions of: (a) brown pigment from wild type eyes taken out by double extraction (curve A); (b) brown pigment from eyes of the mutant *bw* extracted by the same procedure (curve C); (c) brown pigment from the eyes of the same mutant extracted directly in AMA (curve B). It will be noticed that on account

of differences in concentration the ordinates in this figure are given as log E. The three curves have definitely similar shapes.

For reasons which will not be discussed here we chose to use for the suppression of brown pigment the gene *st*. It therefore became important to ascertain whether or not the eyes of the mutant *st* contain quantitatively the full amount of red pigment—that is, whether the gene *st* suppresses the formation of the brown pigment only. Several experiments were performed in which wild type and *st* flies were raised under similar standard conditions (see above). They were grown in the same bottles. Table 5 contains the data obtained. It may be seen that the pigment content in *st*, as compared with wild type is 99.4 percent



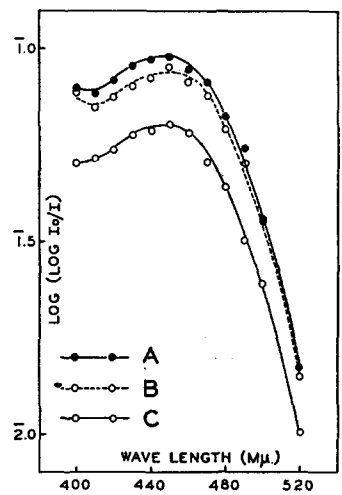


FIGURE 18 (left).—Absorption curves of red pigment extracted from identical numbers of eyes of wild type, *cd*, *st*, *cn*, and *v* ♀ ♀ (Coleman).

FIGURE 19 (right).—Absorption curves of brown pigment extracted A—from wild type ♂ ♂ by "double" extraction, B—from *bw* ♂ ♂ by direct extraction in AMA, C—from *bw* ♂ ♂ by "double" extraction. E calculated per 10 heads in 1 cc of AMA (Coleman).

in ♂ ♂ and 97.4 percent in ♀ ♀ when calculated from the E values and respectively 97.7 percent and 101.2 percent when calculated from the "Average E" values. In these experiments a systematic difference in weight appears to exist between the flies of the two genotypes. The correction by weight slightly exaggerates the difference in pigment content of females. After correction, the pigment content in *st* ♂ ♂ is 101.8 percent and in *st* ♀ ♀ 105.7 percent of that in the wild type. The value of the correction by weight has already been discussed. It is clear that the contents of red pigment in *st* and wild type are practically equal. It must be emphasized, however, that the two stocks were not isogenic.

The question as to the relative amounts of brown pigment in the eyes of wild type and of the mutant *bw* must also be raised. In order to answer it, it is necessary to estimate the brown pigment in the wild type eye; the difficulties of this estimation are those discussed in the section on double extraction.

TABLE 5

Photometric determination (Pulfrich, 470 mμ) of red pigment in wild type and scarlet flies grown in the same bottles. E—extinction per ten heads in 1 cc AEA.

EXP. NO.	WILD TYPE				SCARLET			
	E	AVERAGE E	WEIGHT OF ♂♂ IN MGS.	CORRECTED AVERAGE E	E	AVERAGE E	WEIGHT OF ♂♂ IN MGS.	CORRECTED AVERAGE E
				♂♂				
IV-171	4.160	4.160	1.072	3.884	4.068	4.068	1.052	3.868
V-1	4.124	4.124	1.024	4.028	4.004	4.004	0.984	4.068
V-5	4.348	4.348	1.020	4.264	4.184	4.184	0.946	4.424
V-13	4.328	4.328	1.090	3.976	4.048	4.048	0.972	4.168
IV-69	3.892				4.132			
	3.928	3.948	0.984	4.012	4.104	4.068	1.018	4.000
	4.024				4.076			
	m=4.115	m=4.182	m=1.038	m=4.033	m=4.088	m=4.074	m=0.994	m=4.106
				♀♀				
IV-171	4.624	4.624	*	4.312	4.484	4.484	*	4.264
V-1	4.404	4.404	*	4.300	4.572	4.572	*	4.648
V-5	4.568	4.568	*	4.480	4.644	4.644	*	4.908
V-13	4.656	4.656	*	4.276	4.528	4.528	*	4.656
IV-69	4.328				4.632			
	4.404	4.348	*	4.416	4.616	4.640	*	4.560
	4.316				4.676			
	m=4.471	m=4.520		m=4.357	m=4.593	m=4.574		m=4.607

* See corresponding experiment in upper part of the table.

It will be recalled that during double extraction a part of the brown pigment is lost, but whether or not the same amount of brown pigment is lost in the extraction of *bw* and wild type eyes is not known. Table 2 contains the data on brown pigment obtained by double extraction of wild type and *bw* eyes and by direct AMA extraction of *bw* eyes. If the amounts of pigment extracted from wild type and *bw* eyes by double extraction are compared, it appears that *bw* eyes contain approximately 30 percent less pigment than eyes of wild type. In this connection it will be noted that wild type and *bw* eyes, after the extraction with AEA is terminated, both appear dark purple, but that the color is definitely darker in wild type. If, on the other hand, the comparison is made between the amount of brown pigment extracted from wild type eyes by double extraction and the amount of the same pigment extracted from *bw* eyes directly with AMA, the difference found is much smaller (6 percent). The important point is that even in this case direct extraction of *bw* eyes with AMA *does not give more pigment* than double extraction of wild type eyes. Even if the loss of pigment by double extraction of wild type is not what it is in the case of *bw*, there is no reason to assume that there is no loss at all, and thus the conclusion is reached that the eyes of the mutant brown contain less brown pigment than those of wild type. Here again the reservation must be made that the stocks used were not isogenic.

RECAPITULATION

The experiments reported above provide the basis of a technique for pigment extraction and measurement which is currently used in this laboratory. This technique is briefly as follows:

Flies grown under "standard conditions" are aged after emergence for at least two days in the case of *bw* and at least five days in the case of *st*.

The flies are decapitated, and the heads are placed in the solvents. Entire *bw* heads are used, while *st* heads are split into halves with a safety razor blade.

Acid ethyl alcohol is used for the extraction of red pigment; acid methyl alcohol for the extraction of brown. The first of these solvents is perfectly stable. The acidity of the second must be frequently checked.

The extractions of pigment in both these solvents are complete in less than 24 hours at 25°C.

The red pigment extract, eventually filtered, may be used without further treatment for photometric determination of the absorption. The brown pigment must be oxidized with hydrogen peroxide. One and one-half to two hours should be allowed for the oxidation at 25°C.

The optimum number of heads per sample depends of course on the instrument used for the determination of absorption. Using a Pulfrich photometer with 50 mm micro-cells the highest accuracy was found at 470 mμ with extracts from 40 *st* heads in 100 cc AEA and from 40 *bw* heads in 10 cc AMA. These numbers of heads are sufficiently great for individual differences to be cancelled.

This technique fulfills the requirements defined at the beginning of this paper. It is a rapid procedure, permitting work with small amounts of material and involving a minimum of manipulation.

DISCUSSION

The various observations made in the course of this technical study of pigment extraction are in agreement with the general conclusions of MAINX and BECKER. The relative genetic and physiological independence of the two pigmentation processes, controlled by the genes *st* and *bw*, is paralleled by the clear differences in the properties and behavior of their end products. This conclusion, in which we claim no originality, is emphasized here because a different picture is frequently assumed on the basis of SCHULTZ'S 1935 paper. To quote only one example, we read in GOLDSCHMIDT'S book (1938, p. 32): "Both the red and yellow pigments are water soluble and may be separated chemically, and the red pigment can be oxidized into the yellow one. Reciprocally, the tan pigment of the early pupa may be reduced to red by action of H_2S. This means that only one pigment, in different states of reduction, is present." Similar statements are found in WADDINGTON'S book (1939) and elsewhere.

The present work, actually based on the different solubility of the two pigments, shows equally clear differences in their behavior with respect to pH and

oxido-reduction changes. The latter observations would leave no doubt that the brown and red pigments are not related to one another as simple oxidation-reduction products were nothing known of their genesis. The relative independence of the two pigments is further emphasized by their different variations with temperature and with the age of the flies.

The independence of the two pigment components is qualified here as relative, because gene mutations at certain loci can suppress, partially or totally, both components simultaneously. This seems to indicate a common step in the reactions leading to the formation of the two pigments. The present work has shown that while the gene *st* almost entirely suppresses the formation of the brown component without interfering with the formation of red pigment, the gene *bw* totally suppresses the formation of red and at the same time somewhat decreases the amount of brown formed. The nature of the common step in the two pigment forming reaction chains is unknown. A possible interpretation has been proposed by EPHRUSSI and CHAVAIS (1938), and new experiments concerned with this problem will be presented in the next paper of this series.

A last remark concerns the "age change." This work has shown that, while most morphogenetic processes in flies are terminated at the time of emergence, the pigment deposition in their eyes is far from complete at this time. It will be recalled that, according to MAINX (1938), the age change concerns mainly, if not exclusively, the brown pigment component. The data presented above show that there undoubtedly is a change in the concentration of both pigments. That this quantitative change accounts for the total age change is doubtful. The visible change in the mutant *bw* seems to extend beyond the 24 hour period during which the increase of pigment concentration may be observed. Although the state of oxidation of the extracted pigment does not necessarily reflect the state of the pigment *in vivo*, the perfectly similar shapes of the curves of figure 13, for untreated and oxidized pigment extracts, seem to indicate that oxidation or reduction are not responsible for the visible change. It is possible that the latter depends, in addition to the increase of pigment concentration, on a change in the state of aggregation of the pigment, due to increase of size of the pigment-carrying granules, as described by COCHRANE (1937).

SUMMARY

A detailed study is made of a technique for extraction and photometric estimation of the red and brown pigment components of the eye of *Drosophila melanogaster*.

The action of some factors of variability of the pigment content (culture conditions, age of flies, etc.) is analyzed.

The pigment content of the mutants *st* and *bw*, used for the suppression of the red and brown pigments, respectively, is compared with that of wild type. The gene *st* is found to suppress almost entirely the formation of brown without interfering with the formation of red. The gene *bw* totally suppresses the formation of red, but also somewhat decreases the amount of brown.

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