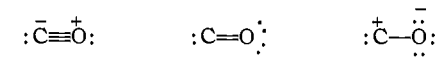
may be indicated by :C≡O: or as resonance among

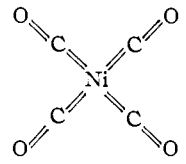


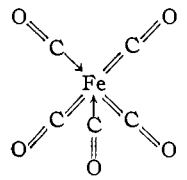
However, Long and Walsh[6] have challenged this and advocated the use of only a double bond structure.

Since bond character is not a direct experimental quantity, conclusions with respect to it naturally vary according to the relative importance an author gives to the various pertinent experimental quantities. Some have given most weight to the bond distances. We would urge that more attention be given to bond stretching force constants as they become available. With caution with respect to dynamic interactions, vibration frequencies may be taken as approximate measures of force constants (together with masses, of course). Thus the frequencies of single, double and triple carbon–carbon bonds lie near 1000, 1650 and 2050 cm.$^{-1}$, respectively. For carbon–oxygen bonds the values are usually a little higher, organic carbonyl frequencies being 1700–1800 cm.$^{-1}$. The carbon monoxide frequency at 2168 cm.$^{-1}$ is thus in the triple bond range. The corresponding frequencies for Ni(CO)$_4$ and Fe(CO)$_5$ are near 2030 cm.$^{-1}$ which indicates that the carbon–oxygen bond, although intermediate, is nearer that in carbon monoxide than that of a ketone or aldehyde.

It has also been suggested that the metal-to-carbon bonds are something approaching double bonds in these carbonyls. The observed frequencies correspond, however, to M–C force constants[16] of about 2.5 × 10$^5$ dynes/cm. which are about the same as for metal alkyls in this region of the periodic table.[17] This tends to indicate that the M–C bonds are not far from single bonds in agreement with the near triple bond C–O frequency.

One particular conclusion of Long and Walsh can be tested. Their structures for Ni(CO)$_4$





and for Fe(CO)$_5$ show the former to have strictly double Ni=C bonds whereas the Fe–C bonds are resonating with 1$^3/_5$ average order. Thus the Ni–C force constant should be distinctly higher. If any difference is noted, it is in the opposite direction, the skeletal stretching frequencies in iron carbonyl being considerably higher than those of nickel carbonyl.

## Summary

Infrared spectra in the region 2–23$\mu$ are reported for the iron carbonyls, Fe(CO)$_5$ and Fe$_2$(CO)$_9$. Some frequency assignments are made. The data on iron carbonyl are fully consistent with the reported trigonal bipyramid structure. The spectrum of iron enneacarbonyl was obtained only for the solid and is relatively meager. However, the bridged structure involving ketone-like carbonyl groups is strongly supported by the observation of a strong band at 1828 cm.$^{-1}$ in addition to the usual metal carbonyl C–O frequencies near 2000 cm.$^{-1}$. The implications of the results in terms of bond types are discussed briefly.

(16) Ref. 13 for Ni(CO)$_4$ and calculations based on the present data for Fe(CO)$_5$.

(17) For example, 2.39 × 10$^5$ in zincdimethyl; H. S. Gutowsky, *J. Chem. Phys.*, **17**, 128 (1949); additional values to be summarized by R. K. Sheline.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND OF BIOLOGY, UNIVERSITY OF OREGON]

# Partition Chromatography of Red Eye Pigment of *Drosophila melanogaster*[1]

BY HANS HEYMANN, FRANK L. CHAN AND CLARENCE W. CLANCY

The red pigment in the eyes of *Drosophila melanogaster* has been shown[2,3] to consist of several colored constituents by means of chromatography of eye extracts on talc columns with the aid of alkaline developing agents. Whereas the brown pigment component of wild-type eye color appears to be related to tryptophan *via* kynurenine,[4] and is apparently melanic in nature,[5] no specific precursor for the red pigment has as yet been demonstrated, and the chemical nature of the red bodies remains obscure. Lederer,[6] on the basis of an elementary analysis of a preparation of not specified purity from wild-type flies, regarded the substances as related to the pterines, a conclusion with which Maas[3] disagreed because he found a much lower amount of nitrogen (6%) in the mercuric chloride precipitate of a purified fraction.

The present work was undertaken in an endeavor to find (a) a reproducible method of chromatographic fractionation of the red pigment complex preliminary to its systematic application to a pigmentary analysis of the various eye color mu-

(1) This work was carried out under a contract between the Office of Naval Research and the University of Oregon.

(2) Wald and Allen, *J. Gen. Physiol.*, **30**, 41 (1946).

(3) Maas, *Genetics*, **33**, 177, (1948).

(4) Beadle and Tatum, *Am. Naturalist*, **75**, 107 (1941).

(5) Tatum and Beadle, *Growth*, **6**, 27 (1942).

(6) Lederer, *Biol. Rev. Cambridge Phil. Soc.*, **15**, 273 (1940).

tants of Drosophila; (b) a method of preparing at least one of the fractions of the red pigment complex in sufficient quantity for eventual chemical identification. The mutant *vermilion* was used as a source of pigment for this study.

## Methods of Extraction

Dialysis of whole or macerated flies[3] from cellophane bags against 30% aqueous ethanol approximately 0.01 *N* in hydrochloric acid (apparent *p*H 2–3)[7,8] was used routinely to extract the crude pigment. Extracts of whole flies (300,000 animals per 4,000 cc.) reached maximum color intensity after about three to four weeks and contained generally about 260 mg. % of dry solids, as weighed after lyophilization. Extraction of macerated flies proceeds more rapidly[3] and solutions of tinctorial power comparable to that of whole-fly extracts contain only about 170 mg. % of dry solids. The absorption spectrum of whole-fly extracts closely resembles that of extracts of heads alone.

Maceration of flies in the presence of water-saturated phenol is an alternate, efficient way of extracting the color, but the large quantities of undesired matter extracted simultaneously discouraged us from further development of this method.

The aqueous alcoholic extracts may be freed of ethanol by vacuum distillation from a bath at 40 to 50°,[3] and of acid by passage through Amberlite IR[4]-B, without loss of color intensity, although a trace of a green-fluorescent and apparently innocuous body is imparted to the solutions by the deacidification.

## Methods of Concentration

An early observation made on material adsorbed on and eluted from talc indicated solubility of the pigments in water-saturated butanol-1, and the possibility of partition chromatography on water-containing silica gel[9] was recognized. However, the dry solids as obtained by lyophilization of extract solutions cannot be dissolved in wet butanol because the presence of a large amount of hydrophilic colorless companion substances invariably leads to the formation of an aqueous phase underneath the butanol, and the pigments, being hydrophilic themselves, dissolve in this rather than in the organic phase. Several methods of concentration were investigated and five criteria employed to judge the success of the procedures: (1) The tinctorial power at 485 mμ was used as a measure of pigment recovery, determinations being carried out with a Beckman spectrophotometer. As Fig. 1 shows, Beer's law holds satisfactorily at this wave length. Unfortunately, the photometric analysis cannot disclose preferential removal of one of several pigments with closely neighboring absorption maxima, and we have tried to guard against such an error by relying on criterion (5), below. (2) In many cases the reduction in weight of the color-containing fraction was noted. (3) Unless interfering substances are introduced the ratio of the optical densities at 265 and 485 mμ is seen to decrease as purification progresses. In the crude extracts the ratio $D_{265}:D_{485}$ ranges from 5 to 10 (see Fig. 1); the purest materials we have handled gave values of about 0.5. One also observes that the maximum in the ultraviolet gradually shifts from 265 mμ toward 270 mμ as purification progresses. The decrease in the ultraviolet absorption coupled with the disappearance of phosphorus from the samples (the crude dry solids contain 0.5% of phosphorus) indicates that probably nucleotides are among the contaminants. (4) The solubility of the purified material in wet butanol was observed. (5) The number and nature of colored regions on the partition column was noted and the results of the several methods were compared *inter se*.

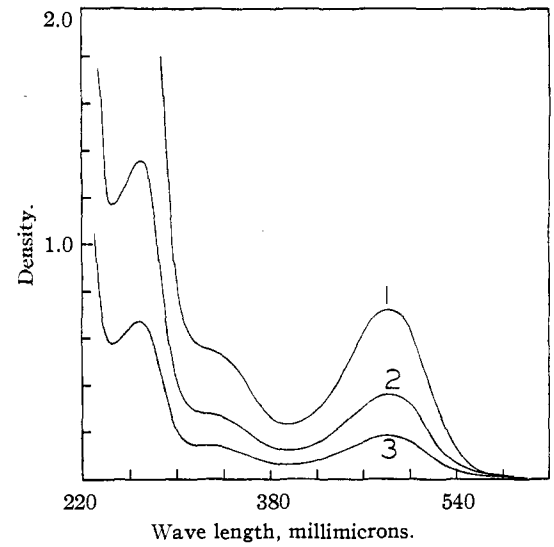


Fig. 1.—Absorption spectrum of *vermilion* eye extract: 1, dilution 1:2; 2, dilution 1:4; 3, dilution 1:8.

A number of approaches were found to be inexpedient. Exposure of the dry solids in ethereal suspension or methanolic solution to diazomethane led to no improvement of solubility. Phenol extraction of aqueous solutions is wasteful of colored material. Silica gel will adsorb the pigments from aqueous media but large amounts of adsorbent are required. Phosphotungstic acid precipitates the pigments,[2] but we found no way of releasing them from the complex in a form suitable for chromatography.

Amberlite IRC-50, a polycarboxylic acid resin, adsorbs the pigments when in the proton form, and on elution with 0.1 *N* hydrochloric acid the recovery of tinctorial power was 53%, the ratio $D_{265}:D_{485}$ was about 2.1, the solubility in butanol was greatly improved, and seven colored regions were easily observable on the partition column.

When the dry solids were treated for twelve

(7) Clancy, *Genetics*, **27**, 417 (1942).
(8) Ephrussi and Herold, *ibid.*, **29**, 148 (1944).
(9) Gordon, Martin and Synge, *Biochem. J.*, **37**, 80 (1943).

hours at room temperature with 1–2 molar anhydrous methanolic hydrogen chloride, the recovered, butanol-soluble material represented 35% of the original tinctorial power; the ratio $D_{265}:D_{485}$ was about 3.5. On partition analysis five to seven well-defined bands were seen, but a slow yellow band otherwise noted was weak or absent; also the appearance of a new, fast, pink band was noted in a few experiments.

A gentler and more efficient procedure utilizes precipitation of the pigments with silver nitrate, after the extracts have been freed of alcohol and acid. The color is released on treatment with methanolic methyl iodide and the pigment can be reprecipitated with ether after removal of the silver iodide. At this point the colored material generally weighed less than 1% of the original dry solids; the color recovery was about 66% and the ratio $D_{265}:D_{485}$ was 1.0–1.5. The material was readily soluble in wet butanol, but for the production of good bands on the partition column an additional reprecipitation with ether from 0.1 to 0.01 *N* methanolic hydrochloric acid was required.

These is no evidence that the pigments have undergone a chemical change during this silver treatment, such as the expected methylation of acidic –OH or –NH groups. The spectrum appears to be unaltered; the color changes, from yellow to orange to red, still occur as one goes from *p*H 1 to 7 to 9; and, under the influence of an electric field (see below) the treated pigments do not behave markedly different from the crude material. The purified pigments, however, cannot be completely reprecipitated with silver nitrate, which might, indeed, indicate methylation of a salt-forming group. On the other hand, since pigment preparations obtained by procedures excluding the possibility of such methylation likewise fail to give silver precipitates, we believe that the initial precipitation involves adsorption of the pigments on other, precipitable silver salts, from which the color is released when they are decomposed by methyl iodide; in this process many of the companion substances are transformed into ether-soluble derivatives and are thus separable from the colored substances.

To a solution of 120–150 mg. of dry solid extractives in 5–7 cc. of water was added about 5 cc. of 0.05 *N* silver nitrate solution. This amount generally causes complete precipitation. The voluminous precipitate was centrifuged, washed several times with water, and suspended in a mixture of 5 cc. of dry methanol and 5 cc. of methyl iodide. After standing overnight the practically colorless silver iodide was centrifuged, washed twice with methanol, and the combined methanol solutions were diluted with at least five volumes of ether. The precipitated pigments were centrifuged, washed twice with ether, dried, and dissolved in 2 cc. of 0.1 *N* methanolic hydrochloric acid. Renewed precipitation with ether yielded the pigments in the form of a yellow-orange gelatinous mat; the solid was dried and dissolved in 3 cc. of water-saturated butanol-1.

The original fly extract may be used directly after removal of the alcohol in vacuum at 40–50° followed by deacidification on Amberlite IR4-B. In the cases where extracts from macerated flies were used, the supernatant above the silver precipitation often showed small amounts of color, whereas with whole-fly extracts the removal of color from solution was complete.

Colored material in the ethereal supernatant after the methyl iodide treatment sublimed partly when the ether was distilled (traces of iodine) and such pigmented material as remained in the distillation residue had no defined absorption maxima in the visible region.

Charcoal strongly adsorbs the pigments from aqueous solutions and the adsorbate may be washed extensively with water and solvents, prior to elution with phenol which is an efficient agent for this purpose. The recovery of tinctorial power was 74%, and at the end of the processing 64% of the total tinctorial power was recovered in the wet butanol solution. Ten colored regions were observed in the chromatogram.

A 180-cc. sample of eye extract was freed of alcohol by vaccum distillation at 40 to 50° which reduced the volume to 106 cc. After dilution of an aliquot to five volumes the spectrum was determined; $D_{265}$, 1.85; $D_{485}$, 0.170. The solution, still containing acid, was passed through a 20 by 20 mm. column of a 1:1 mixture of charcoal (Darco) and Supercel (diatomaceous earth) which rested on a small mat of Supercel. The weight of the charcoal-Supercel layer was 2 g. All of the color was retained on the column which was then washed with 75 cc. of water, 40 cc. of acetone, 40 cc. of 0.1 *N* methanolic hydrochloric acid, 20 cc. of acetone and 20 cc. of methanol. As the last washing ran through, color appeared in the filtrate. The column was eluted with 25 cc. of water-saturated phenol and the eluate, together with the last methanol wash, was evaporated *in vacuo* at 40–50° until the water was removed, leaving 15 cc. of a deep red phenol solution. After addition of dry ether to a volume of 80 cc. the reddish flocculent precipitate that formed was centrifuged, washed with ether, dried briefly, and dissolved in 0.1 *N* methanolic hydrochloric acid with brief warming. A small amount of insoluble brown gelatinous solid was centrifuged off, and an aliquot of the solution was precipitated with ether for spectrophotometry. The ratio $D_{265}:D_{485}$ was 2.4 but this figure is of doubtful value in view of the uncertainty as to complete removal of the highly ultraviolet-absorptive phenol. The bulk of the pigment solution was precipitated with ether and the dried solid taken up in a little water to which dry butanol was added until the aqueous phase just disappeared. A small amount of brown scum present was removed by centrifugation and the volume brought to 10.0 cc. with wet butanol. An aliquot, precipitated with ether and made up to 20.0 volumes with water, gave the values: $D_{265}$, 0.377; $D_{485}$, 0.230; corresponding to 64% recovery of tinctorial power.

In another, larger experiment 1000 cc. of fly extract was reduced to 350 cc., and passed through a charcoal-Supercel column 100 by 19 mm. The column was washed with 100 cc. of water and 100 cc. of methanol, and eluted with 70 cc. of phenol. The butanol solution eventually resulting from this treatment was used in the chromatographic experiment to be described below.

**Partition Chromatography.**—After silica gels prepared from water-glass[9] had proved promising as media for partition chromatography using wet butanol as the mobile phase, a larger supply of suitable silica was obtained from the Mallinckrodt Chemical Company. Mr. W. M. Kelley of that Company kindly furnished three samples of different history to permit us to select the most suitable one; it appeared that for our purposes there were but small differences among the three batches of Silicic Acid, Mallinckrodt.

The silica gel is heated overnight at 150° and stored in a tight container. Seventy per cent. by weight of butanol-

TABLE I

PARTITION CHROMATOGRAM ON A COLUMN 310 BY 23 MM. OF 15 CC. OF BUTANOL SOLUTION CONTAINING PIGMENTS FROM 485 CC. OF VERMILION EYE EXTRACT

Legend: p, purple; y, yellow; o, orange; r, red; c, carrot. Underneath each entry of time and volume passed are tabulated the locations of the regions observed, given in mm. counted from the top of the silica column, the visual appearance of the regions, and, in the last four columns, the zone numbers as used in Fig. 2.

| Time developed, hr. | 1.5 | 3.5 | | 8.5 | | 15[a] | | 23 | |
|---|---|---|---|---|---|---|---|---|---|
| Volume passed, cc. | 20 | 42 | | 95 | | 168 | | 255 | |
| Appearance of column | 3–5 p | 8–10 p | | 14–16 p | | 19–21 p | | 22–23 p | |
| | 5–16 o | 12–16 oy | 1 | 18–19 p | | 23–26 p | | 32–34 p | |
| | 18–23 or | 16–20 c | 2 | 21–27 y | 1 | 31–41 y | 1 | 42–58 y | 1 |
| | | 20–22 or | | 28–33 c | | 43–52 c | | 60–65 c | 2a |
| | | 26–29 or | 3 | 34–36 or | 2 | 53–58 or | 2 | 68–75 c | 2b |
| | | 32–35 oy | 4 | 37–38 o | | 61–63 o | | 78–82 or | 2c |
| | | 38–40 or | 5 | 44–49 or | 3 | 68–70 o | | 85–90 o | 2d |
| | | | | 50–52 o | | 72–80 or | 3 | 95–99 o | 3a |
| | | | | 56–63 oy | 4 | 82–86 o | | 107–113 o | 3b |
| | | | | 68–72 or | 5 | 92–99 o | 4 | 119–124 o | 3c |
| | | | | | | 111–122 o | 5 | 134–143 o | 4 |
| | | | | | | | | 160–180 o | 5 |

[a] At this time development of the parallel column was interrupted and regions 3, 4 and 5 were sectioned for spectrophotometry.

saturated water is well mixed in. After the moistened but still powdery gel has cooled, it is suspended in wet butanol (2–3 cc. per g.) and poured into an adsorption tube; a sintered glass plate or a plug of glass wool serve as retainers. Nitrogen pressure is applied (5–14 lb. per sq. in.), the gel is allowed to settle, and the excess butanol is filtered through. The size of a settled column, which will not shrink any further under suction or pressure, that can be obtained from 10 g. of dry gel is about 70 by 19 mm.

When a layer of butanol about 3 mm. thick is still left on the surface of the column, the pigment solution is added very carefully so as not to deform or disturb the surface of the rather pliable gel column. After percolation of the solution development with wet butanol is begun. A rate of flow of about 0.06 cc. per sq. cm. of column surface per minute was found to be advantageous, and can generally be attained by means of a pressure of 4–8 lb. per sq. in.; very long and narrow columns require higher pressures. Suction is not suitable because it causes evaporation of the solvent, and cracking of the column, at the lower end. After development, the column is sucked dry, *i. e.*, until no more liquid percolates out under suction, pushed out and sectioned. Elution is accomplished with 0.1 *N* methanolic hydrochloric acid, or, advantageously, with water-saturated phenol in porous-bottom gooch crucibles which may be centrifuged and cause a minimum of silica to appear in the eluate. The pigments may be precipitated with ether and generally contain traces of silica; the only means to remove this contaminant appear to be extensive centrifugation or, better, dialysis of a concentrated aqueous solution followed by lyophilization.

The course of a chromatogram on a column 310 by 23 mm. in which one-half of the butanol solution described above was used is shown schematically in Table I. Since the fast-moving bands become diffuse before the slower ones are fully developed, two columns were used in parallel, one of which was cut apart when the fast bands were clearly visible while the other one was developed further to secure the slower regions.

One first observes that a colorless, diffuse fraction, recognizable by its strong blue fluorescence in ultraviolet light (Corning filter H. R. Red Purple Ultra no. 587), detaches itself from the colored section and moves toward the filtrate. With concentrated solutions a weakly greenish zone is also seen, as well as a diffuse and very weakly orange region, both of which move rapidly along the column and run out with the filtrate. The colored region at the top of the column is then seen to break up into a strongly colored section of orange hue, and into a faster moving, more reddish portion. This faster moving portion soon breaks up into three discrete regions, labeled 3, 4, 5 in the Table; of these, region 3 eventually gives rise to three discernible sections. No further subdivision of regions 4 and 5 could be observed. Region 1 likewise appears to consist of a single entity of clear yellow appearance. At the end of the development region 2 has broken up into four distinct zones, of which the second one, the carrot-red band (2b) is by far the most strongly colored band on the entire column. Above the bands described one generally notices one or two weak brownish-purple regions which move very slowly and which seem to be different in nature from the main bands just described; possibly they represent brown transformation products of the kind that Maas[3] has mentioned.

Although the colored zones are clearly distinguishable from one another, they are merely regions of maximum accumulation rather than of exclusive retention of a given component; thus most of the interstices between regions were not entirely pigment-free, and in sectioning only the heart-portion of each colored zone was selected. For the purpose of comparison, the eluates were dissolved in known volumes of water prior to spectrophotometry, and Fig. 2 represents the absorption spectra of the eluates corresponding to the ten regions shown in Table I; the curves are comparable with one another with the exception of curve 2b for which the ordinate should be multiplied by two. About 52% of the total tinctorial power present in the original butanol solution was recovered in the eluates; part of the loss is due to

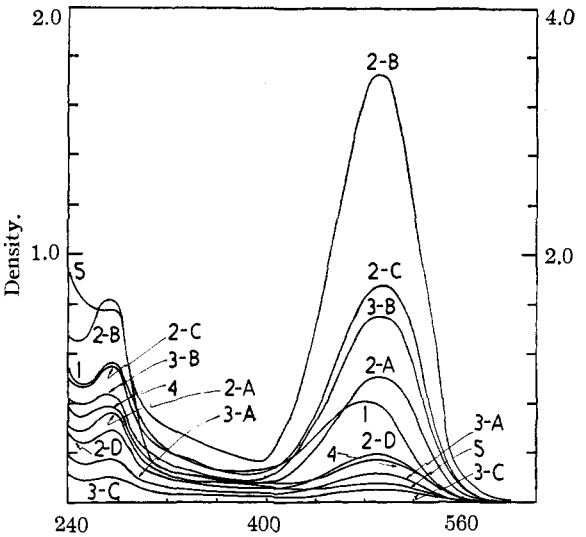


Fig. 2.—Absorption spectra of components of *vermilion* complex.

the imperfect separation of the bands as well as to incomplete elution. The carrot-red band (2b) contains more than 50% of the total tinctorial power present on the column. In the group labeled 3 the middle zone (3b) is the strongest, amounting to about 12% of the total color, and the yellow band (1) contains about 5% of the color. The quantitative relations among the weaker bands are subject to doubt.

All of the zones described have absorption maxima within the range, 480–490 mμ. As pointed out above they differ in relative abundance, and it also seems that the ratio between the densities of the ultraviolet and the visible maxima may be a characteristic property of the constituents; thus, this ratio was 0.54 for the carrot-red band of the fifteen-hour column noted in Table I. The eluate was rechromatographed in a duplicate experiment, giving rise to a large, homogeneous carrot-red band with only a very small amount of one or two other colored regions visible. The color recovery was 45–57%, although it should be noted that complete elution was not attempted. The density ratio after rechromatographing was 0.51, 0.48 The values of this ratio for the other constituents have not been sufficiently studied to date.

Some interaction seems to occur between silica, butanol and the pigment, for when band 2b of the twenty-three-hour column noted in Table I was eluted immediately after sectioning and examined spectrophotometrically the ratio $D_{275}:D_{490}$ 0.48 was obtained. On the other hand, when the silica gel containing the pigment was allowed to stand for ten days in the cold, the eluted and rechromatographed pigment gave a value of 0.61 for this ratio, and the color recovery was rather poor. Probably this interaction is responsible for some loss of tinctorial power even during the relatively short period of development.

The representation of the chromatogram containing ten zones is based on observations of about 20 to 30 columns, using the various methods indicated for purifying the crude material. It should be noted that the very weak bands are not seen if too small a quantity of pigment is employed, and we explain our failure to find more than seven or eight bands in some of the runs by noting that in those cases the amount of pigment analyzed was very small. The silver method, the IRC-50 method, and the charcoal–phenol method give essentially the same chromatograms. There is reason to believe that in the hydrogen-chloride method some material is destroyed, and some bodies are formed that were not originally present, as indicated above. Finally, one must note that the amount of colored material must not be too large lest one overtax the capacity of the column and the material appears in the filtrate before sufficient development has taken place.

Attempted paper chromatography failed because butanol or quinoline did not cause the firmly adsorbed pigments to migrate, whereas phenol as the moving phase gave no fractionation.

We have endeavored to establish connection between our bands and those seen by Wald and Allen,[2] and by Maas[3] on the talc column; and have obtained by talc chromatography samples of the topmost, reddish band, and the second, peach-colored fraction, which we believe to be zones I and II in Wald's terminology. When these colors were chromatographed on silica, zone I gave rise to a sharp carrot-red band which we believe to be the main band (2b) in our second group of pigments. Zone II from the talc column gave rise to a large yellow band on the silica column, no doubt the same as the yellow band labeled 1 in Table II. There was also noted a faster-traveling orange-yellow band, which we cannot place with certainty in its relation to the total partition chromatogram. There exist a number of discrepancies between our results and those of earlier reports. Both Wald and Allen,[2] and Maas[3] found that the largest amount of red color was contained in the number II zone on talc, whereas our carrot-red band (2b) seems to correspond to the topmost region (zone I) on the talc column. Moreover, all of the bands we observed have absorption maxima between 480 and 490 mμ; we saw no pigments in our chromatograms that had maxima at 420 mμ or at lower wave lengths in the visible region such as were reported in both of the papers cited. Possibly, the reason for these differences is that we used pigment derived from the mutant, *vermilion*, whereas Wald and Allen[2] did not include this mutant in their investigation; Maas[3] employed the mutant *cinnabar* routinely but stated that he obtained the same chromatogram from *vermilion* flies. We carried out one analysis on *cinnabar* and were unable to detect profound differences be-

tween the *cinnabar* and the *vermilion* pigments, but a small variation may exist. Although we did lose a definite amount of color in our procedures, we cannot believe that the same group of pigments should have escaped us in every one of three or four different concentration methods. We cannot, at this time, suggest a wholly satisfactory explanation for the discrepancies mentioned.

The appearance of as many as ten distinguishable colored regions on the partition column is surprising and suggests the question whether some of them might not be artefacts formed during processing or even during chromatography itself. The behavior of band 2b on rechromatography argues against the latter possibility. There is no reason to question the specificity of the partition method used, *i. e.*, no reason to believe that it will lead to accumulation of one and the same chemical in more than one zone on the column unless one assumes that through some agency, *e. g.*, colorless companion substances, the column is rendered inhomogeneous, an assumption that does not appear reasonable in view of the results of the silver method in which removal of more than 99% of the colorless by-products was ascertained. Further, the reproducibility of the chromatograms following application of quite different procedures is an argument against secondary formation of some of the fractions observed, but a fully convincing proof that all ten of them are present in the original extract will require further investigation.

Table II summarizes the results of a number of simple electrophoretic tests, in which small amounts of pigment solution were inserted in the middle of potassium chloride–agar bridges. The ends of the bridges were protected from direct contact with the fluids near the electrodes by immersing them in beakers containing potassium chloride solution, and from these separate agar bridges led to the electrode compartments. It appears clear that the colored substances exist as cations in acidic medium, as anions in base, and that they are neutral molecules or zwitterions near neutrality.

A larger sample of purified pigment was prepared by adsorption on silica gel from water, elution with hydrochloric acid, deacidification, extraction with phenol and ether precipitation, followed by several reprecipitations from methanolic hydrochloric acid and glacial acetic acid. Thus from 300,000 flies, 74 mg. of a dark red, hygroscopic powder was obtained, which, at the concentration 0.018 mg. per cc., had the optical densities $D_{270}$, 0.690; $D_{485}$, 1.27. The pigment concentrate contained chlorine and nitrogen; sulfur was absent, and phosphorus was no longer detectable. The halogen is ionic and some of the observations reported above suggest that the colored molecules may exist in the form of hydrochlorides in the concentrates. (The method of extraction would, of itself, lead to the formation of hydrochlorides of basic extractives.) Even though the optical data indicate that considerable purification has been achieved, the absence of non-colored companion substances is not established. We hope that the present methods will eventually lead to the crystallization in pure form of at least one of the colored fractions for quantitative analysis.

TABLE II

ELECTROPHORESIS BEHAVIOR OF *Vermilion* PIGMENT SOLUTION IN ACIDIC, NEUTRAL AND BASIC MEDIUM

| Medium *p*H | Pigment motion toward | Distance moved, mm. | E. m. f. across bridge, volts | Current, mA. | Time exposed, hr. |
|---|---|---|---|---|---|
| 2.6 | − | 25 | 100 | 60 | 3.0 |
| Same | − | 15 | 32 | 60 | 5.3 |
| 3.15 | − | 25 | 50 | 60 | 8.0 |
| 2.8[a] | − | 250 | 320 | 10 | 3.0 |
| 8–9.4 | + | 6 | 94 | 60 | 2.0 |
| Same | + | 30 | 48 | 70–99 | 5.2 |
| Neutral | Spread evenly | | 28 | 60 | 7.3 |
| Neutral | Very little | | 0 | 0 | Overnight |

[a] This run was carried out with glass wool plugs rather than agar to prevent diffusion; nine pieces of glass wool were distributed along the bridge.

## Summary

Methods of concentrating the eye extracts from *Drosophila melanogaster* are described. By partition chromatography with wet butanol on silica gel the existence of ten closely related entities has been shown. Their absorption spectra have been recorded. The charge-type variation with *p*H of the pigments has been described.

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