PHENOL OXIDASE CHARACTERISTICS IN MUTANTS OF DROSOPHILA MELANOGASTER[1]

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SINCE the very early experiments of GRAUBARD (1933), numerous investigators have examined the problem of genetic control of phenol oxidases in Drosophila with attention focused primarily on pigment production and thus on the color mutants. However, it has become clear that the function of such enzymes in cuticle formation is far more vital than in pigment production. It follows that if the same enzymes are involved in both processes, and this now seems highly probable, then mutations to enzyme loss would be lethal and mutations to pigment alterations must be due to modest alterations of structural proteins or entirely due to regulation of function. At present the latter interpretation appears the more attractive, particularly in view of the probability that the phenol oxidases play a dual role, having catalytic function in oxidation and also a function as material providing fixed macromolecular structure.

Early evidence that can be interpreted in this direction (HOROWITZ and FLING 1955) demonstrated that Drosophila extracts contain no phenol oxidase function prior to an incubation period to permit activation, and that after activation the product with enzyme function is easily sedimented by centrifugation. More recently (MITCHELL and WEBER 1965) it has been shown that at least five proteins are involved in the processes of phenol oxidase activation, and orderly aggregation of several different protein subunits to yield very large and complex structures has appeared as the best interpretation of the data. This postulation now receives further support from the data presented here on the macromolecular nature of the phenol oxidases produced in extracts of the Oregon-R wild strain of *Drosophila melanogaster* and in extracts of four pigmentation mutants.

Based on velocity and equilibrium sedimentation studies in sucrose gradients, *in vitro*, activation to produce phenol oxidase activity yields at least seven active components relatively low in density but very large in size. Extreme and significant differences exist among the mutants and wild type.

MATERIALS AND METHODS

Stocks and culture: The Oregon-R wild stock has been used throughout these studies. The mutant strains used were from the stock center maintained at the California Institute of Technology and have the following constitutions: yellow (*y*); Blond (*Bld*) maintained heterozygous as *T(1:2)Bld/ ClB;* ebony-11 (*e¹¹*); black (*b*).

The wild type and mutants were all cultured on a large scale for selection of animals in

developmental synchrony as described previously (MITCHELL and MITCHELL 1964; MITCHELL 1966). Under these conditions no significant differences in developmental rates were noted among the stocks used. The straw mutant used previously was not included in these experiments since it presents technical difficulties in mass culture.

Enzyme preparation and assay: Although a number of substrates and methods of assay for phenol oxidases have been employed during this investigation, virtually all of the data presented are based on initial rates of dopachrome production from dopa as described previously (MITCHELL 1966). Reactions were carried out in a Cary recording spectrophotometer at 30°C and measurements were made at a wave length of 475 mμ. Rates are all reported in terms of optical density change per 10 minutes calculated from one-minute scans.

In earlier work enzyme activation was carried out in cell free but crude extracts of whole animals and, as noted, maximum activities achieved represented a summation of activation and inactivation rates. The inactivation aspect of the system has now been virtually eliminated by a partial purification of the whole system and the standardized preparation of enzyme used in these experiments is illustrated by the following example. All operations were carried out at temperatures below 4°C.

A uniform sample (2 g) of animals frozen at —80°C was mixed with sand (1 g) and 4 ml of 1.5 M KH_2PO_4 in a 600 ml mortar in an ice bath. The sample was ground thoroughly with slow addition of 26 ml of ice water. Insoluble material was removed by centrifugation at 0° at 55,000 $\times$ g. The supernatant solution was brought to 56% saturation by addition of cold saturated $(NH_4)_2SO_4$ made up in 0.1 M phosphate buffer (pH 6.3). The precipitate was washed into a single tube with 56% saturated $(NH_4)_2SO_4$ and pelleted in the centrifuge (78,000 $\times$ g for 5 min). The residue was then dissolved in 1.5 ml of 1.5 M KCl in 0.02 M phosphate buffer (pH 6.7) and any insoluble residue removed by centrifugation for 20 minutes at 105,000 $\times$ g. This solution contains approximately 10% (30 mg) of the original soluble protein but all of the original activation potential of the crude extract. No activation occurs in the presence of KCl at this concentration (1 M urea or 0.1% sodium dodecylsulfate can also be used), but a slow loss in potential occurs after several hours in the high salt solution.

For activation the KCl solution was immediately passed through a 6 ml bed volume of Sephadex G25 (fine) prepared in 0.02 M phosphate buffer at pH 6.7. Only the excluded protein (2.4 ml following the void volume) was collected. By the end of this operation, which requires about 15 minutes, most samples were activated to about half maximum. Routinely, activation was followed for 2 to 3 hours using 5 μl aliquots in 0.6 ml of substrate solution (0.0025 M dopa in 0.1 M phosphate buffer at pH 6.3). Maximum activity for most preparations was achieved within 60 minutes and it remained constant for several hours with usually a loss of less than 10% in 24 hours. However, slow aggregation occurs, and for all of the experiments described here activation times of 2 to 3 hours were used.

Sucrose gradients: All gradients were linear and made from reagent grade sucrose dissolved in 0.02 M phosphate buffer (pH 6.7) using glass mixing vessels in the cold. To insure reproducibility, sucrose solutions were weighed into the mixing vessels. Densities were determined by direct weighing of aliquots.

For sedimentation velocities the 5 to 20% sucrose system of MARTIN and AMES (1961) was used and for sedimentation equilibria light and heavy solutions were prepared by dissolving sucrose in the amounts 0.7 g and 1.8 g, respectively, per 1 ml of buffer (densities 1.190 and 1.310 at 4°C). Nearly all of the data given here are for 30 ml gradients run in the SW25 rotor of a Spinco L-2-65 centrifuge, although the SW65 and SW25.2 rotors were used for some special purposes. For 30 ml gradients 1 ml fractions were collected after puncturing the tube with a No. 25 syringe needle.

All of the sedimentation equilibrium experiments described here were carried out at 25,000 $\times$ g (max) and at this gravitational force ultimate sharp bands in 16 to 20 hour equilibrium experiments are already clearly established in 3 hours. Also at this speed more than 90% of the phenol oxidase activity applied to a gradient can be recovered. However, it is important to note that at higher speeds, and even at this speed in an angle rotor, phenol oxidase activity is irreversibly lost. At the extreme of 350,000 $\times$ g in an SW65 rotor only 1.5% of the starting activity remained

after a 2-hour run. Extensive investigations with variations in ionic strength, cations (Mg, Ca, K, Na) and sucrose-Ficol combinations failed to demonstrate conditions under which activity could be preserved at high speeds.

RESULTS

Total activities: As shown in Figure 1 the wild-type Drosophila and the mutants studied each has its own characteristic pattern when the age of animals is related to maximum phenol oxidase activity. All of the mutants possess a much higher potential for activity than normal in late third instar larvae but the subsequent disposition of potentials follows different courses. For example, the γ mutant loses virtually all of its potential activity by 40 hours after puparium formation whereas the *Bld* mutant retains a high activation capacity throughout pupal life. It may be noted also that in spite of this drastic difference between these two mutants both end up just before emergence with a higher activity than normal. In contrast the corresponding levels for the highly pigmented mutants *b* and *e* are lower than wild type and this difference becomes greater following emergence when pigmentation becomes more intense.

It is of special interest to note the changes in activation capacities in the age range of 55 to 70 hours. The relatively small increase observed with wild type was not apparent in crude extracts (MITCHELL 1966), but its reality is empha-
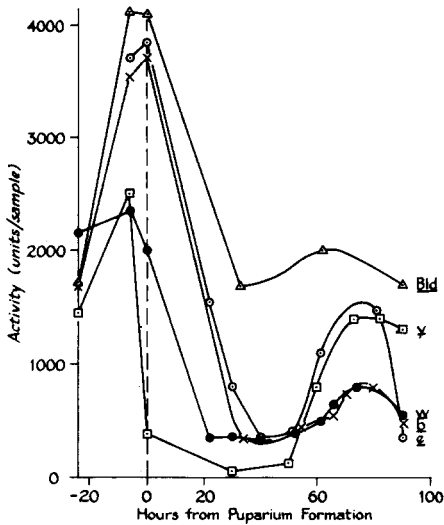

FIGURE 1.—Maximum phenol oxidase activities achieved in purified extracts of different age animal of wild-type (Oregon-R) and mutant stocks of *Drosophila melanogaster*. The range of the life cycle covered is from mid-third instar to four hours before emergence of adult flies.
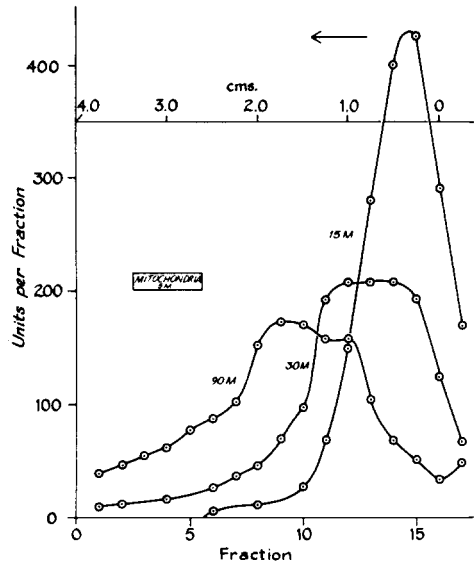
FIGURE 2.—Sedimentation velocity patterns of phenol oxidases prepared from wild type at the time of puparium formation. Distributions of activity in a 4 cm linear gradient of 5 to 20% sucrose in buffer are given for times of 15, 30 and 90 min at 32,000 × g. Under the same conditions mitochondria of Neurospora move to the position indicated in 5 min.

sized here by the extreme changes in the mutants *y* and *e*. It may be recalled that it is during just this period that wild type is sensitive to heat shock treatments to yield *Bld* phenocopies.

Sedimentation velocity: An example of a sedimentation velocity pattern of Drosophila phenol oxidases is shown in Figure 2. As illustrated the largest components sediment about one-third as fast as a sample of *Neurospora* mitochondria (courtesy of Mr. R. T. EAKIN) used for a marker and most of the activity sediments within 1/30 the rate of the most rapid component. These facts, coupled with the lack of sharp bands which would indicate homogeneity with respect to size, strongly suggest enzyme aggregation to different degrees. It may be noted, however, that once formed the material is stable since repeat runs of fractions from velocity gradients yield banding corresponding to the behavior of the fraction examined. In any case, the indication of aggregation has been confirmed by direct observation in the microscope as shown in Figure 3. Activated enzyme without substrate is not visible in the light microscope, but as shown in Figure 3a it can be seen in phase contrast as aggregates of spheres having a diameter of about 1 μ. In the presence of a limiting amount of dopa the enzyme itself becomes pigmented uniformly with only a small loss in activity, and it is then readily visible in the light microscope, as shown in Figure 3b, which shows also an apparent unit about 1 μ in diameter. It should be noted that the material observed here is from component 2 (Figure 7) derived from the mutant *e* and sedimenta-
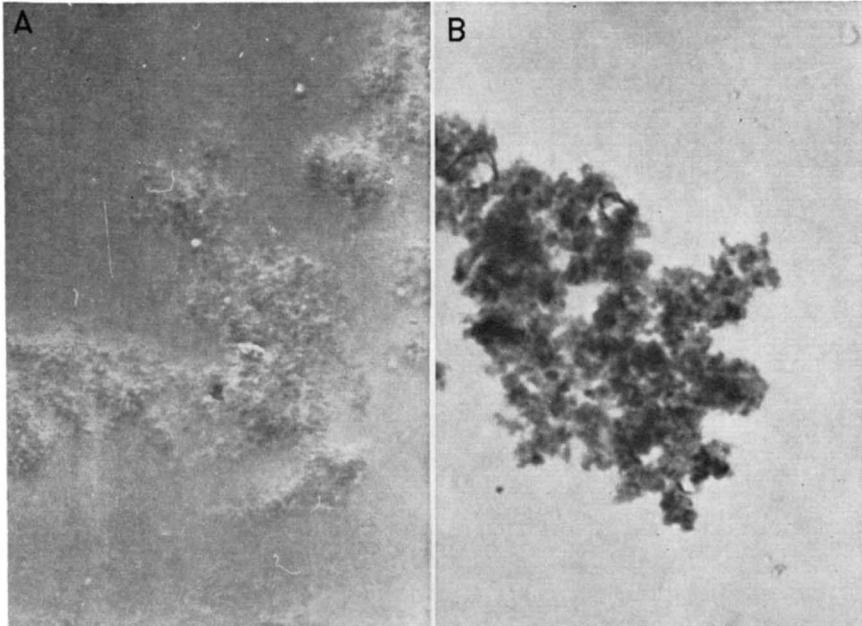

FIGURE 3.—Photographs of banded phenol oxidase component 2 from the mutant *e*. A. Phase contrast without substrate. The material is not visible in light. B. The same material as in A after a 2 hour incubation at 0°C with 1 μg/unit of dopa. Light microscope. In both pictures the small spheres from which the large aggregates are formed are about 1 μ in diameter.
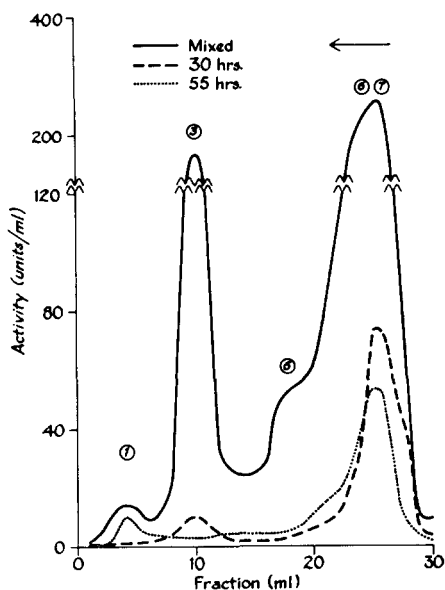
FIGURE 4.—Sedimentation equilibrium patterns of phenol oxidases from Oregon-R wild stock extracts. The mixed sample includes very late larvae and early prepupae and represents the situation just around puparium formation. The other samples are from animals after puparium formation at the ages indicated. The circled numbers (see also Figure 5) show positions of major activity bands.
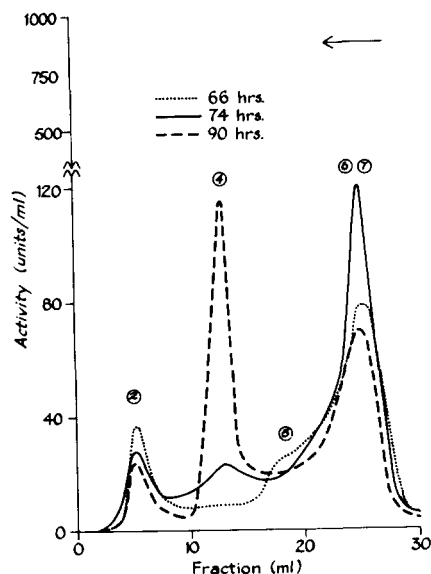
FIGURE 5.—Sedimentation equilibrium patterns of phenol oxidases from Oregon-R wild stock extracts. (See Figure 4 legend.)

tion equilibrium experiments. Components 1 to 5 (Figures 4 and 5), or most of the phenol oxidase material, have a similar appearance when freshly prepared.

Sedimentation equilibria: In contrast to enzyme distribution patterns obtained in sedimentation velocity gradients, those obtained from separations based on density show a high degree of homogeneity. This is illustrated for wild-type preparations from animals of different ages in Figures 4 and 5. Based on these data and others not presented, seven components are indicated as a minimum number of phenol oxidases having different densities that can be formed *in vitro*. Component 6 is the least well defined since it has not been studied in low-density gradients and it may well be subdivided on further examination. However, it should be pointed out that components 1 to 6 oxidize both dopa and tyrosine whereas component 7 oxidases tyrosine very poorly if at all. Furthermore, component 7 contributes at most 2% of the total phenol oxidase activity in larvae and it is considerably smaller in size, having an S value of approximately 20. Details will be presented elsewhere.

With respect to mutants, several examples of distributions of enzyme components based on density are given in Figures 6 to 9. As shown in Figures 6 and 7 extracts of *e*, *b* and *Bld* prepared just at the time of puparium formation (approxi-
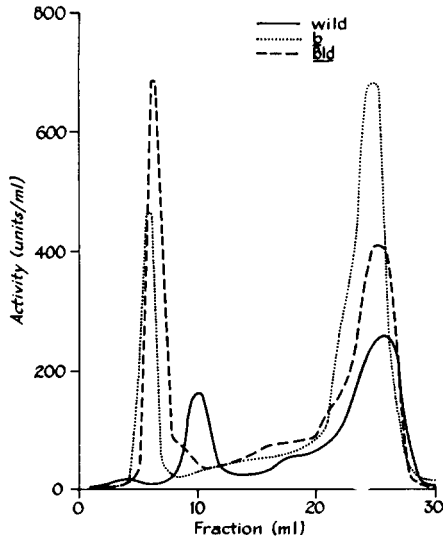
FIGURE 6.—A comparison of sedimentation equilibrium patterns of phenol oxidases from b, Bld and wild type at the time of puparium formation.
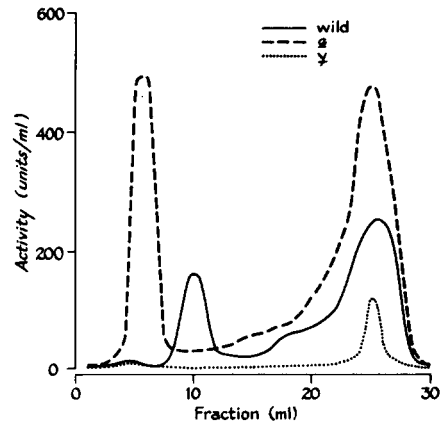
FIGURE 7.—A comparison of sedimentation equilibrium patterns of phenol oxidases from e, γ and wild type at the time of puparium formation.

mately 1:1 mixture of very late larvae and very early prepupae) yield a great excess of component 2 and little or no component 3, which is prominent in the wild-type pattern. That this is the result of rapid changes in potential in the late third instar is illustrated in Figure 8, which shows production of the heavy components 1 and 2 in the larvae of b. Here it is clear that in the period between —24 and —6 hours a change occurs to permit the formation of component 1, and that in the next six hours another change permits the formation of component 2. The mutant e shows a similar pattern, but wild type yields very little of components 1 or 2 at any time in larvae. From these observations it is clear that a large part of the excess activity found in late larval stages in e, b and Bld is due to increased capacities for the formation of components 1 and 2.

In contrast to these pattern characteristics of e, b and Bld, the γ mutant has very little potential for enzyme activity at puparium formation (Figure 7) although it has about a normal amount in late larvae (Figure 1). In this case it appears that the loss in oxidase activation potential which normally occurs after puparium formation takes place too soon.

Some examples of comparative patterns for extracts of late pupae just before emergence are given in Figure 9. Pattern similarities are evident, as are quantitative differences. At this point e lacks component 2 but this is due to a loss in capacity during the last few hours of pupal life (see Figure 10).

For convenience, and since all of the available data cannot reasonably be presented here, summaries of the age distribution of components 2 and 4 are presented in Figures 10 and 11. As shown, the potential for production of component 2 appears just before puparium formation but only in Bld, e and b to any great
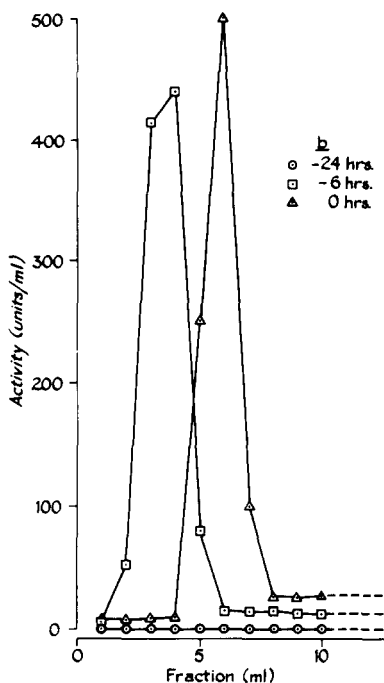
FIGURE 8.—The sequence in formation of phenol oxidase components 1 and 2 during the last day of the larval stage in the mutant *b*.
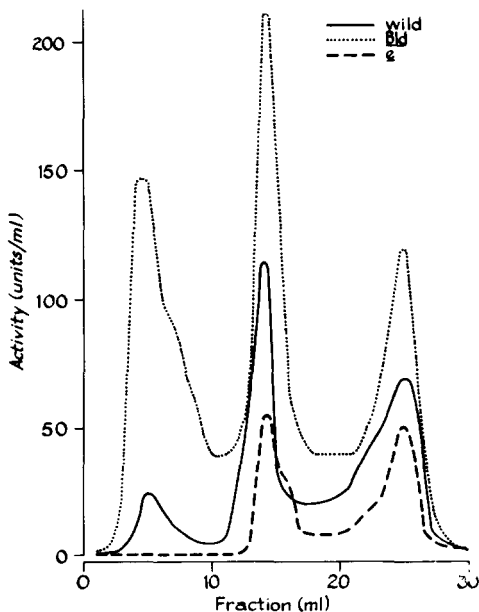
FIGURE 9.—A comparison of sedimentation equilibrium patterns of phenol oxidases from *e*, *Bld* and wild type 90 hours after puparium formation.

extent. Subsequently, the potential appears again in late pupae but the differences in amounts among the strains are much less dramatic. Here again the data emphasize the 55 to 70 hour period as one demanding production of phenol oxidase potential. The same can be said of component 4 (Figure 11) which has been observed only in late pupae. Here there are also marked differences among the mutants examined. This is true also with respect to comparisons of other enzyme components (1, 3, 5 and 6), but it should be noted that, in general, differences are mostly quantitative to the extent of the present analysis, and a very important conceptual aspect concerned with the origins of such differences is illustrated by results from enzyme activation in samples mixed prior to activation.

Composite activation: As mentioned in connection with sedimentation velocity experiments, once it is formed, activated enzyme retains its sedimentation behavior, and this is also true in density equilibrium gradients. Thus, mixtures of formed enzyme behave additively. However, if mixtures are made before activation, the resulting patterns are not the sum of those obtained from individual samples. Two examples of this important phenomenon are given in Figures 12 and 13. In these experiments, in order to assure adequate controls, extracts from animals of two different ages or from two genetically different stocks of the same age were individually prepared to the KCl stage of purification before activation.
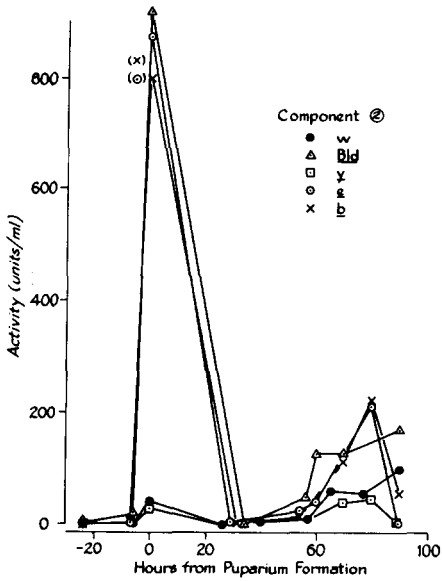
FIGURE 10.—The relative amounts of phenol oxidase component 2 at different stages of development in mutants and wild type.

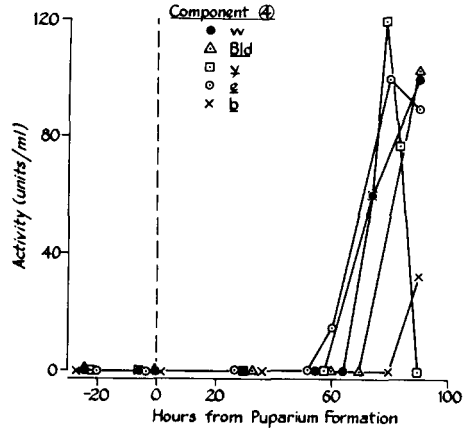FIGURE 11.—The relative amounts of phenol oxidase component 4 at different stages of development in mutants and wild type.
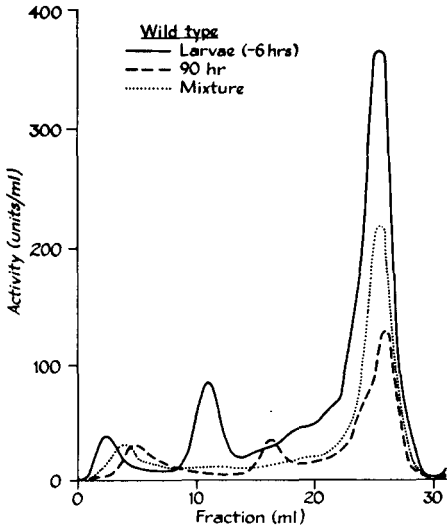

FIGURE 12.—Phenol oxidase patterns from activation in a composite mixture and in unmixed samples from two developmental stages of Oregon-R.
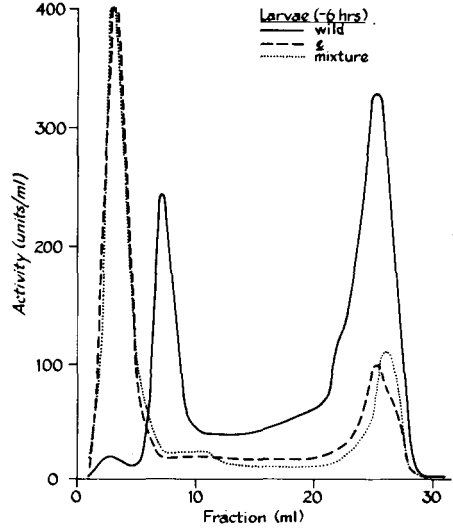

FIGURE 13.—Phenol oxidase patterns from activation in a composite mixture and unmixed samples from late larvae of wild-type and the *e* mutant.

One third of each was taken and mixed to form a composite sample. Each of the three samples was then desalted, activation was followed to completion and all three were subjected to density gradient separations simultaneously. As shown in Figure 12, the composite sample from wild-type late larvae and 90-hour pupae yielded no heavy bands characteristic of either sample alone. Even more striking were the results from a composite sample from third instar (−12 hours) wild and e larvae as illustrated in Figure 13. At this age, component 1 predominates in e and component 3 is prominent in wild type, but as shown, the activity distribution in the composite sample is virtually identical with that from the e mutant alone. Thus, in this case, material from the extract of e completely dominates the activation process in the mixture with the wild-type extract. Rates of activation did not differ significantly, and at present there is no reason to attribute these results to more than differences in concentration ratios of protein components in the extracts prior to activation.

<center>DISCUSSION</center>

From the data presented it is clear that extreme differences exist among the mutant and wild type stocks of Drosophila that have been examined with respect to the quantity and quality of phenol oxidases produced in extracts (Figures 1, 6, 7). From the genetic standpoint it is quite possible that some of these differences may be due to the influence of modifying genes such as have been described by Lewis and Lewis (1963) rather than to the specific mutant loci designated as b, e, γ and Bld. With the present information on specific differences this question can now be approached reasonably, but it is far from a simple problem since the differences are a function of the stage of development. Only in Bld (Figure 1) is there an excessive quantity of phenol oxidase production in extracts from most stages of development and in this case high values are consistent with results previously obtained with blond phenocopies (Mitchell 1966). Since isogenicity is assured in the phenocopy situation there is no reason to expect modifier gene function in the Bld mutant results. With respect to the other mutants, all resemble wild type and each other at some stage of development and if modifying genes are functioning they must be regulatory. Actually from a consideration of the whole system it seems just as likely that the e, b, γ and Bld genes are themselves regulatory.

Considering now the general picture of factors concerned with phenol oxidase formation it is apparent from the data in Figures 1, 10, and 11 that one especially important period in the synthesis of components necessary for enzyme production is in the last half of the larval third instar, and a second is in the range of 55 to 72 hours after puparium formation. This does not mean that synthesis does not occur at other times, only that it seems to be the greatest during these periods. That it is synthesis that occurs in the late larval period is indicated strongly by the changes in the distribution of phenol oxidase components shown in the example given in Figure 8. Here it is evident that a synthesis had to occur between −24 and −6 hours, and another between −6 hours and the time of puparium formation. A number of other similar situations have been observed both in this

period and in the 55–72 hour period. As to the losses of activation potential such as the very large one just following puparium formation (Figures 1, 4 and 5) this is likely due to self inactivation after enzyme formation in the production of cuticle and pigment in the pupal case. Similarly, the losses, especially in *e*, *b* and wild just before emergence, are probably due to like functions. It should be noted that activation potential levels off just prior to emergence, and the differences are greater after emergence, being low in *e* and *b* and high in *y* and *Bld*. This is consistent with the observations of GRAUBARD (1933) and with the self-inactivation characteristic of phenol oxidases in general.

With regard to the macromolecular nature of the phenol oxidases obtained *in vitro* from various ages of wild type and mutants, it is clear from the pictures in Figure 3 that aggregation occurs during activation. Measurements of sedimentation velocities, such as those shown in Figure 2, give little information other than the suggestion that the aggregates are quite stable. This is evident from the fact that reruns of fast and slow fractions give repetitive behavior. Much more significant evidence suggesting dependence of phenol oxidase activity on large particle size and perhaps asymmetry, comes from the fact of inactivation by centrifugation at higher speeds. Material such as that shown in Figure 3a loses 98.5% of its activity in the few minutes required to sediment it in a swinging bucket rotor at 348,000 × g, and about 50% during sedimentation at 80,000 × g. On the other hand, such material is stabilized to centrifugation by reaction with enough dopa to give some melanization, a little corresponding increase in density, and the appearance shown in Figure 3b. Such results suggest association of enzyme activity with a long chain or fiber, with stabilization by quinone crosslinking following reaction with substrate, but no firm conclusion can yet be drawn from preliminary studies of structure. In any case, these indications of very large molecules are in accord with previous evidence that the phenol oxidases are produced by orderly aggregation among at least five protein components (MITCHELL and WEBER 1965; MITCHELL 1966; GEIGER and MITCHELL 1966). From the results described here on products from activation in composite samples (Figures 12 and 13) it appears, furthermore, that the kinds of products obtained in any activation are dependent on the proportions of protein components present. A general working hypothesis which can account for the various equilibrium density gradient patterns observed from extracts of different stages and mutants is shown in Figure 14. The formation of products of different density, of course, must be accounted for, and it may be noted that all are lighter than would be expected for a complex of pure protein since enzyme component 1 (the heaviest) has a density close to 1.30. Although lipid contents have not been determined, indirect evidence is available that protein components A_2 and S are lipoproteins and a participation by lipid in activation has previously been noted by LEWIS and LEWIS (1963). Thus lipid may account for at least part of the density differences among the heavier enzyme components where gradient equilibrium is assured.

One further interesting question that should be mentioned concerns the possible biological significance of the seven or more enzyme components of different densities, but nothing more than speculation can be offered since no formed enzyme

PHENOL OXIDASE FORMATION

Protein Subunits	Protein Components	Enzyme Subunits	Enzyme Aggregates
2 $\longrightarrow$	A_1		
? $\longrightarrow$	A_2		
? $\longrightarrow$	A_3		
? $\longrightarrow$	P		
? $\searrow$ PS $\nearrow$	S		

A_x
P_y
S_z

At least seven
combinations with
definite proportions
and densities

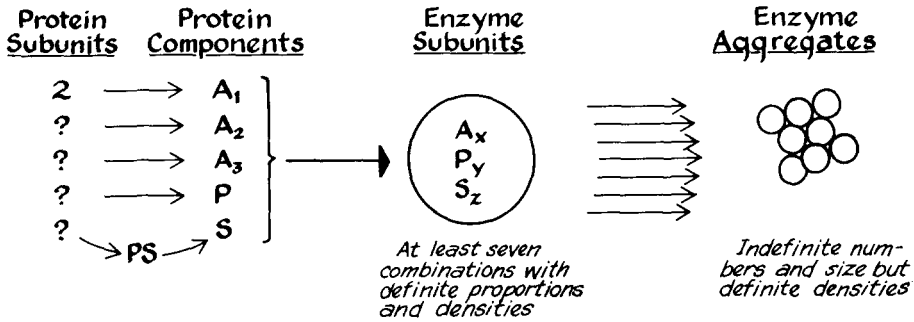Indefinite num-
bers and size but
definite densities

FIGURE 14.—An hypothesis describing the origin of the seven or more phenol oxidase complexes that are obtained *in vitro*.

has been extracted from living material. Evidently, *in vivo*, when phenol oxidase is formed it is immediately fixed as part of insoluble structure and is eventually inactivated through its own functions. Such a unique property seems evident from observations of uniform self-melanization of enzyme as illustrated in Figure 3. This situation, as well as the overall picture, appears to be quite analogous to the well documented (see MOYER 1966) description of the formation of mammalian phenol oxidase as part of the structure of melanin granules. In this latter case the very large and complex structure, of which the enzyme is a part, has been well defined by electron microscopy, and it has been proposed that at least three different protein components are required for formation. It is possible that the Drosophila enzymes are similar in structure to long (0.5–0.7 μ) double helical protein chains with periodic active centers, characteristic of the single units of mammalian premelanin granules, but it is not likely that the higher order granule structure is attained. In Drosophila the phenol oxidase function is far more vital and varied than just in pigment formation since it is essential in the formation of cuticle, adrenalin and possibly resilin, as well as pigment.

SUMMARY

Freshly prepared extracts of the Oregon-R strain or four mutant strains (e, b, γ and *Bld*) do not contain phenol oxidase activity, but the function appears on incubation at 0°C. The potential for maximum activity is achieved in all strains around the time of puparium formation, followed by a loss in potential and then a second maximum about one day before emergence. Active enzyme is largely particulate in nature and is irreversibly inactivated by high speed centrifugation. At low speeds seven active enzyme components can be defined by equilibrium sedimentation in sucrose density gradients. Characteristic patterns of these components are found at different stages of development and in different mutants. However, patterns of active products are clearly dependent on extract composition with respect to the protein components from which phenol oxidases are

formed. The data suggest that the phenol oxidases are highly asymmetric protein complexes which may serve as structural protein as well as oxidative enzymes.

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