STUDIES ON THE A COMPONENTS OF

DROSOPHILA PHENOL OXIDASE

Thesis by

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This thesis is

dedicated to my

parents.

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ABSTRACT

In insects, the enzyme phenol oxidase is involved in the hardening and darkening of the cuticle. Drosophila phenol oxidase occurs in a latent form. Phenol oxidase activity is produced as a consequence of the interaction of several proteins. Three of these proteins are designated as the A components (A_1, A_2, and A_3). A quantitative assay for the A components has been developed. The A_1 component has been prepared in stable and highly purified form by gel filtration, preparative electrofocusing, and preparative electrophoresis. No major contaminants could be detected by analytical polyacrylamide gel electrophoresis, analytical electrofocusing, SDS gel electrophoresis, and analytical ultracentrifugation. The A_1 component has a molecular weight of approximately 77,000 daltons and an isoelectric point of 5.1. The isoelectric point of the A_2 component is 6.0, and its molecular weight is similar to that of the A_1 component. The A_1 component has no detectable phenol oxidase activity and cannot be converted to active phenol oxidase by the S (salivary gland) component. The A_1 component contains approximately 0.15% copper and does not contain large amounts of fatty acids or phosphate. The amino acid composition of A_1 is reported. Microheterogeneity was observed in preparations of purified A_1. The relationship of the A components to active phenol oxidase and the mechanism of the activation

reaction are discussed.

CONTENTS

INTRODUCTION

Adaptations involving the insect integument depend upon precise developmental control of morphology, toughness, pigmentation, and permeability. The great success of insects is associated with the evolution of a vast variety of cuticular structures. It is therefore not surprising that intricate and tightly regulated enzyme systems have evolved to meet the strict demands of cuticle synthesis. Insect cuticle is characterized by a layer, the epicuticle, composed of protein covalently crosslinked by oxidized derivatives of phenolic compounds (1). The enzyme phenol oxidase catalyzes the oxidation of phenolic substrates by molecular oxygen. The resultant quinones are exceedingly reactive. They can enter into polymerization reactions and into crosslinking reactions with cuticular proteins. As a consequence, the cuticle acquires pigmentation and rigidity. This thesis will be concerned with the complex phenol oxidase system of Drosophila melanogaster. In order to provide a background for the discussion of Drosophila phenol oxidase, the following introduction will consider the comparative biochemistry of phenol oxidase with special emphasis on insect phenol oxidase and its relation to cuticle structure. Note will also be taken of the frequent instances where organisms are protected against the extreme reactivity and even toxicity

of quinones by maintaining unneeded phenol oxidase in a latent form.

Nomenclature and Reactions.

The enzyme activity which is identified as phenol oxidase in this thesis has been called tyrosinase, phenolase, phenolase complex, dopa oxidase, polyphenol oxidase, cresolase, and catecholase by various authors (12). The systematic name is o-diphenol: O_2 oxidoreductase (E.C. 1.10. 3.1), and it is to be distinguished from the similar enzyme, laccase, or, p-diphenol: O_2 oxidoreductase (E.C. 1.10. 3.2) (2).

Phenol oxidase catalyzes two distinct reactions. The first is the oxidation of o-diphenols to o-quinones, sometimes referred to as the catecholase activity. The second is the ortho hydroxylation and oxidation of monophenols, sometimes referred to as the cresolase activity. The ability of a single enzyme to catalyze two different reactions has intrigued biochemists for over thirty years and has led to extensive research on phenol oxidase.

Fungal Phenol Oxidase.

At the present time, the phenol oxidases of the common edible mushroom have been studied in greatest detail. Their properties are similar to the phenol oxidases which have been subsequently examined, and most data pertaining to the

question of reaction mechanism come from this system. Consequently, it will be useful to examine the mushroom enzyme in some depth. The mushroom phenol oxidase literature has been reviewed several times; unfortunately none of these reviews are recent (3, 4, 5, 6, 7).

Over the years a number of laboratories have contributed purification schemes for mushroom phenol oxidase. Early schemes based primarily on salt and organic solvent fractionation techniques have been improved by the introduction of DEAE-cellulose and hydroxylapatite chromatography (8, 9, 10, 11, 12, 13, 14, 15, 16, 74). Although some conflicting results have appeared, most of these conflicts are now resolved, and a fairly coherent summary may be presented.

Mushroom phenol oxidase preparations contain four isoenzymes named, according to Bouchilloux et al., α, β, γ, and δ (8). They can be resolved on DEAE-cellulose (13) and hydroxylapatite (17). The β, γ, and δ forms share a sedimentation constant of 7.2s, while the α form sediments at 6.7s. From sedimentation and diffusion measurements, the β, γ, and δ forms have a molecular weight of 123,800 daltons. Kertesz and Zito reported a somewhat smaller s value for their preparations ($s_{20,w}$=6.3), but the calculated molecular weight of 128,000 daltons is similar (10). Frieden and Ottesen agree with the larger value ($s_{20,w}$=7.3) (9). When sedimentation of β phenol oxidase was carried out in SDS, a 2.57s peak appeared suggesting dissociation of a tetramer

(124,000 daltons) into a monomer (31,000 daltons). Under some chromatographic conditions, the monomer (17) and dimer (15) may be isolated directly. The monomer-multimer equilibrium has been studied by starch gel and polyacrylamide gel electrophoresis, as well as analytical ultracentrifugation (18). Although aggregates up to the dodecamer have been observed, the tetramer is the dominant species observed for the β-isozyme. The equilibrium is strongly dependent on protein concentration. Dissociation is promoted by SDS, urea, EDTA, succinylation, acetylation and iodination. Although a claim has been made that the monomer is enzymatically inactive, the question of the enzymatic activity of the various associated forms cannot be resolved on the basis of the present evidence (19). In general, the authors cited above agree that mushroom phenol oxidase contains about 0.2% copper, corresponding to one copper atom per monomer.

The origin and significance of the phenol oxidase isozymes is a perplexing problem. The first major difference between them, other than the chromatographic behavior which permits their separation, is substrate specificity. The ratio of specific activities for p-cresol and catechol as substrates varies over two orders of magnitude for different fractions (8, 17). This variability does not merely result from a preponderance of o-diphenol oxidase activity over monophenol hydroxylase activity in certain fractions; the effect can be reversed by using other substrate pairs.

Thus α phenol oxidase has a much lower specific activity with catechol than γ phenol oxidase, but with DOPA as substrate α phenol oxidase has a higher specific activity than the γ form. Similarly, Long et al. found that their isozyme fractions were distinguishable not only by the p-cresol to catechol specific activity ratios, but also by the ratios for 3,4-dimethylphenol and p-cresol, DOPA and p-cresol, and catechol and DOPA (13). A more subtle difference between the isozymes was uncovered by Dressler and Dawson who studied the exchange of phenol oxidase copper with radioactive copper (20, 21). All preparations failed to exchange copper in the absence of substrate, but in the presence of substrate, the high catecholase samples exchanged copper freely while the high cresolase samples exchanged at a much lower rate. The α, β, γ, and δ isozymes are barely resolved by analytical acrylamide gel electrophoresis (17). The β, γ, and δ isozymes have an identical or nearly identical amino acid composition. Moreover, their tryptic peptide maps are indistinguishable. All four contain only a single N-terminal amino acid, isoleucine, and a single C-terminal amino acid, valine. The α isozyme is the only one with a distinct chemical composition, having higher threonine, proline and tryptophan content. It will be recalled that the α form also has a lower sedimentation constant. However, the differences in substrate specificity between the β, γ, and δ forms cannot clearly be explained in terms of protein structure.

Two classes of explanations for the multiple forms may be considered on the basis of the available data. The first is genetic heterogeneity, an attractive possibility which has been confirmed for Neurospora phenol oxidase and which Fling et al. suggest may account for multiple forms in mushrooms (22). There is no real genetic information on the commercial mushrooms generally used. Single amino acid substitutions which might affect enzyme activity could have gone undetected in the analyses mentioned above. The second class of explanations is modification during purification. Some of the forms may be derived from one native precursor by chemical modification, partial denaturation, or subunit exchange. In this regard it is worth considering some of the peculiar properties of phenol oxidase which complicate its manipulation in the laboratory. Phenol oxidase can act on tyrosyl residues in peptides (75) and enzymes (23, 24) with, in some cases, alteration of the catalytic properties of the oxidized proteins. It is possible that during isolation phenol oxidase might act in this fashion on itself, thus producing artefactual derivatives. In the presence of substrate, a condition which is unavoidable in the early stages of any phenol oxidase purification, enzymatically generated quinones may react with the enzyme. This is the generally accepted basis for the commonly observed phenomenon of phenol oxidase inactivation during the oxidation reaction. This concept has been supported experimentally by Wood and

Ingraham who showed that phenol oxidase covalently binds 1-^{14}C-phenol during oxidation (25). Nakamura reported the separation of two phenol oxidase fractions on DEAE-cellulose one of which was substantially darker than the other (15). He suggested that it had reacted with quinonoid substances during isolation. Thus phenol oxidase represents an unusual case of an enzyme which can be modified and even inactivated by the products of its own activity. Other mechanisms for generating artefactual isozymes such as partial denaturation or subunit exchange between true isozymes can be considered, but no evidence is available to support these possibilities.

The final topic to be considered in regard to mushroom phenol oxidase is reaction mechanism. It has proven difficult to construct a model for both the mono- and diphenoloxidase activities which is fully consistent with the experimental data. The problem still cannot be regarded as near solution. The earliest studies of phenol oxidase revealed the key role of copper (11, 26, 27). Removal of copper leads to inactivation which can be reversed by cupric ions. Assuming the copper to be in the cupric state, a reasonable mechanism involves cyclic reduction and oxidation of copper:

$$\text{Enz}Cu_2^{+2} + \text{o-diphenol} \longrightarrow \text{Enz}Cu_2^{+1} + \text{o-quinone} + 2H^+$$

$$\text{Enz}Cu_2^{+1} + 2H^+ + \tfrac{1}{2}O_2 \longrightarrow \text{Enz}Cu_2^{+2} + H_2O.$$

However, this sort of mechanism has been essentially ruled out by the discovery that the copper in phenol oxidase is in the cuprous state (8, 15, 28). While there are some diffi-

culties in the determination of the oxidation state of copper in cuproproteins, reports from a number of laboratories agree that phenol oxidase copper is in the cuprous state (29). Some cupric copper may occur in aged preparations, but enzyme containing cupric copper appears to be inactive (8, 12).

The following observations place additional constraint on reaction mechanisms. The oldest of these is the lag period observed in the oxidation of monophenols which is absent when diphenols are used as substrates (11, 30, 31). Addition of catalytic amounts of a diphenol to the reaction mixture eliminates the lag period, but this effect, which has been given great weight in the modeling of reaction mechanisms, is non-specific. Other reducing agents, such as ascorbic acid and DPNH also eliminate the lag period. To further confuse matters Karkanis and Frieden have reported that no lag period occurs at all with their purified preparations (32). Furthermore, they found a protein factor which is removed only in the last step of the purification. When added back to pure enzyme this factor restores the lag period and inhibits activity. The inhibitory factor also inhibits activity on diphenolic substrates. No further publications confirm this report.

Another observation relevant to the reaction mechanism was made by Mason *et al.* who used ^{18}O tracer methods to identify the source of the substituent hydroxyl group in the

monophenol oxidation reaction (33). They obtained quite unambiguous results. When $H_2{}^{18}O$ was used in the reaction mixture, no ^{18}O was incorporated into the product. However, when $^{18}O_2$ was used, the oxygen incorporated into the product was labeled. Considering the possibility that a semiquinone might be the primary reaction product, Mason et al. used an ESR spectrometer equipped with a flow cell to look for free radicals in the reaction mixture (34). The concentration of free radicals found was too low to support a free radical mechanism; those found evidently arise from dismutation of quinones. Wood and Ingraham using tritiated 3,4-dimethylphenol measured a slight but significant isotope effect (35). They conclude that hydroxylation must be a rate limiting step and attribute the low magnitude of the effect to acceptance of the leaving hydrogen ion by a basic group on the protein.

Before proceeding to a consideration of specific models for the phenol oxidase reaction, it is necessary to deal with the relation of the cresolase and catecholase activities of phenol oxidase. In general it is agreed that both of these activities are indeed properties of a single protein inasmuch as highly purified preparations retain both activities. Kertesz has favored the idea that the hydroxylation reaction is altogether non-enzymatic and is brought about by the quinones produced by diphenol oxidation (36). He has adduced kinetic data in favor of this view. In light of the

preparation of specific isozymes with varying cresolase to catecholase ratios, the $^{18}O_2$ experiments cited above, and the stereospecificity of the hydroxylation reaction (37), most workers agree that Kertesz' position is untenable and that phenol oxidase plays a true catalytic role in the hydroxylation reaction. Granting then that both reactions are catalyzed by the same protein, they may occur at either the same active site or at two different active sites. The only data favoring the latter concept are the copper exchange work cited above, and no detailed two site model has been proposed.

Several single site models have been brought forward (38, 4). Most authors assume that the first step in the reaction is the formation of a copper oxygen complex comparable to hemocyanin (43). This concept has been supported by kinetic data (41). Malmström, drawing analogy with copper-benzene complexes, has suggested that the role of copper may be substrate binding not oxygen activation (39). A copper-oxygen complex might hydroxylate monophenols as follows (74):

$$\text{Enz}Cu_2^{+1} + O_2 \longrightarrow \text{Enz}Cu_2^{+1}\text{-}O_2 \longrightarrow \text{Enz}Cu_2^{+2}\text{-}O_2^{-2} \xrightarrow{H^+}$$

$$\text{Enz}Cu_2^{+2}\text{-}OOH^{-1}$$

$$\text{Enz}Cu_2^{+2}\text{-}OOH^{-1} + \text{monophenol} \longrightarrow \text{o-diphenol} +$$

$$\text{Enz}Cu_2^{+2}\text{-}O^{-2}.$$

Oxidation would then proceed:

$$\text{o-diphenol} + \text{Enz}Cu_2^{+2}\text{-}O^{-2} \longrightarrow \text{o-quinone} + \text{Enz}Cu_2^{+1} + H_2O$$

Authors who ascribe importance to the lag period attempt to

explain it by reduction either of copper (4) or of an unknown electron carrier on the protein (39). The first possibility is inconsistent with the observation that the copper is already reduced in the resting enzyme, and the second lacks experimental support. Specific mechanistic models cannot be properly evaluated since there is no direct evidence of copper oxygen complex formation or copper oxidation state change. Even the number of copper atoms per active site is unknown.

Neurospora.

The phenol oxidase of Neurospora crassa has been prepared in crystalline form (22). Its properties generally resemble the mushroom enzyme. The predominant molecular species is a monomer of 31,000 daltons containing a single cuprous ion. The monomer freely undergoes an association-dissociation reaction. There is an interesting report that the Neurospora phenol oxidase is the product of an activation reaction which follows first order kinetics (40). The magnitude of the effect observed was rather small. Only crude extracts were used, and the putative precursor was not isolated. A similar effect was noted in the ascomycete Podospora anserina from which phenol oxidase has been highly purified (42). This phenol oxidase occurs as a latent form which activates during purification. Heating extracts at 60° C. also causes activation. The latent and active forms

have differing electrophoretic mobilities but exhibit the same sedimentation constant. The Podospora enzyme is purified as a monomer of 42,000 daltons which undergoes an aggregation reaction.

Mouse Phenol Oxidase.

In contrast to the mushroom system discussed above, mouse phenol oxidase has been examined in detail at the genetic level but has only recently begun to yield biochemical information. In vertebrates, phenol oxidase activity is limited to the **melanocytes**, a class of differentiated cells characterized by the presence of melanosomes, organelles which are the intracellular locus of melanin production. The electron microscope reveals a complex internal structure within the melanosome (44). Fibrils are organized and crosslinked in a regular folded sheet pattern bounded by a unit membrane. Presumably several proteins other than phenol oxidase are involved in the ultrastructural organization of the melanosome. It would therefore appear likely that mutations affecting these proteins would also affect the organization of the melanosome and the deposition of melanin (47). This concept of melanosome structure has been supported by electron micrographic studies of melanocytes in strains of mice carrying mutations which result in altered pigmentation (44, 45, 46). Thus mutant alleles in the C (albino), B (brown) and P (pink eyed dilution) loci result in characteristic ultrastructural alteration of the melanosome. Evi-

dently the C locus is the structural gene for phenol oxidase itself. This work has been further supported by gel electrophoretic analyses of mutant hair bulb extracts (48). Although isolation and chemical analysis of melanosomes has been attempted, the same structural tightness which has permitted genetic analysis presents severe obstacles to biochemical analysis (49, 50, 51). At the present time no information is available concerning any of the structural proteins of the melanosome other than phenol oxidase. The information concerning phenol oxidase has been meager until very recently. Mouse phenol oxidase has been prepared from melanoma and hair bulb. All authors agree that the majority of mouse phenol oxidase is very tightly particle bound (52, 53). Recent efforts have centered on disruption of the melanosome structure in order to yield a soluble preparation. Detergents, lipolytic enzymes, and sonication release phenol oxidase into solution (53, 54, 55). A soluble preparation has also been obtained from an acetone powder (56, 57, 58, 59). These preparations contain at least three active phenol oxidases which may be interrelated. Like mushroom phenol oxidase, the activity of the mouse enzyme depends upon the presence of a copper prosthetic group (60). The mouse enzyme further shares the property of a lag period in the oxidation of tyrosine which can be shortened by the addition of reducing agents (61). The substrate specificity of the mouse enzyme is narrower than the mushroom enzyme; the pres-

ence of a side chain amino group is necessary (62). Again, as in the mushroom system, melanization of the enzyme leads to inhibition of activity (63).

A very interesting report recently appeared which represents an important step in the understanding of melanosome structure (65). Working with the 20% of mouse phenol oxidase which is readily soluble, and taking advantage of the unusual stability of mouse phenol oxidase in 8 molar urea, Burnett has purified two phenol oxidases to homogeneity by repetitive preparative polyacrylamide gel electrophoresis. The two fractions, T_1, and T_2, have different molecular weights as determined by equilibrium ultracentrifugation (T_1, 66,000 daltons; T_2, 56,000 daltons) and different amino acid compositions. They do not seem to be interconvertible. If one assumes that a single gene, the C locus, codes for phenol oxidase, then the occurence of two distinct proteins must be explained. A reasonable possibility is that T_1, and T_2 are derived from a common precursor, possibly by association with a melanosome structural protein. Burnett calculated the hydrophobicities of T_1, and T_2 which are similar to the figures for the mushroom and Neurospora enzymes.

An interesting regulatory mechanism for mouse phenol oxidase was proposed by Chian and Wilgram (64). They isolated a low molecular weight (less than 5,000 daltons), heat stable inhibitor of phenol oxidase. They suggested that this inhibitor prevents phenol oxidase action until phenol oxidase

is incorporated into the melanosome structure. Some support for this model was provided by examination of two albino melanoma strains isolated from white patches in pigmented melanoma. One of these contained an active phenol oxidase with slightly altered electrophoretic mobility which was evidently inactivated *in situ* by the presence of the inhibitor.

Other authors have considered the possibility that mouse phenol oxidase may be regulated via activation of a latent form in a manner analogous to the well documented insect system (55). There is, however, no evidence of a soluble inactive precursor in mice.

Other Vertebrates.

Phenol oxidase has not been examined in detail from vertebrate sources other than the mouse. Some interesting observations have been made. Electrophoretic examination of hair bulb extracts from black rats, black rabbits, gerbils, and golden Syrian hamsters produced patterns similar to those obtained from mice, suggesting that a protein resembling T_1, is present in many species (66).

In the skin of the newt *Triturus cristatus* two soluble phenol oxidases have been identified (67). One oxidizes only diphenols and is activated by anionic detergents; the other oxidizes both mono- and diphenols and is inhibited by anionic detergents. Activation phenomena have also been

demonstrated in Rana pipiens eggs, where phenol oxidase activity in extracts is enhanced by denaturing agents (68). Adult Rana pipiens skin contains a soluble inactive precursor of phenol oxidase which is evenly distributed over pigmented and non-pigmented skin (69). It can be converted to active phenol oxidase by trypsin. After melanization the active enzyme cannot be extracted.

A number of reports describe the soluble and particle bound phenol oxidase in fish (70, 71, 72). The electrophoretic pattern in one case resembles that found in mice (73).

Insects.

In insects phenol oxidase plays a major role in cuticle synthesis. Although this is not the place for a detailed discussion of cuticle structure and origin, it will be useful to summarize the current knowledge of this topic before taking up specific enzyme systems. Insect cuticle is a layered extracellular structure secreted by an underlying epithelium of specialized cells. Descriptions of cuticle in different species vary, but in general there is a thick inner layer, the endocuticle, composed of chitin, protein, and lipid, and a thin outer layer, the epicuticle composed primarily of protein and lipid (76, 77, 78). Depending on the organism, its stage of development, and the location on its surface, cuticle may vary greatly in hardness and darkness. The

structure of the endocuticle, which has been recently reviewed, will be of no further concern here (79). The epicuticle, however, is the site of phenol oxidase activity. The role of phenol oxidase in cuticle synthesis was first suggested by Pryor who studied the tanning of the cockroach ootheca (egg case) (80). This system was reinvestigated by Brunet (81, 140). Although cockroach oothecae contain no chitin, they harden and darken while in the genital vestibulum of the female, acquiring physical and chemical properties similar to hardened cuticle. The ootheca is tanned by a mixture of the secretions of the right and left collaterial glands. The left collaterial gland secretes an insoluble phenol oxidase and a mixture of the 4-O-β-glucosides of dihydroxybenzl alcohol and dihydroxybenzoic acid. The right collaterial gland secretes a glucosidase, and mixture of the two secretions frees the diphenols for enzymatic oxidation. The resulting quinones react with free amino groups in the oothecal protein and with each other forming the tanned ootheca. Pryor named the quinone tanned protein sclerotin and suggested that a similar process occurs generally in insect cuticle (82). Dennell was the first to provide evidence for this model in cuticle (83). He found that sections of larval cuticle from Sarcophaga falculata darkened when placed in solutions of tyrosine or catechol. The darkening originated in the epicuticle and diffused inward into the endocuticle. This process resembles the normal

formation of the puparium. Pryor's suggestion has received considerable experimental support and has been extended to crustacea (84, 85, 86) and even to other invertebrate phyla (87).

Several major problems emerge at this point. What is the nature and origin of the tanning enzyme, and how is its spatial and temporal distribution controlled? What is the substrate, the tanning agent, and how do the reactions of quinones lead to hardening and darkening (88, 90)? If both hardening and darkening proceed through basically the same mechanism, can they be under independent control? Finally, what controls precisely timed developmental events such as tanning of the fly puparium or the newly emerged adult (89)? Of these, only the first is of direct relevance here. The others are discussed in detail in the reviews cited above.

Melanoplus.

The first insect phenol oxidase to receive much attention was that of the grasshopper Melanoplus differentialis. Bodine and coworkers published an extensive series of papers describing some aspects of the Melanoplus phenol oxidase (91, 92, 93, 94, 95, 96). The most important result of their work is the observation that in Melanoplus eggs, phenol oxidase occurs as an inactive precursor. If fresh extracts are not centrifuged, activation occurs without additional treatment. Activation is accompanied by

aggregation, and the active enzyme can be removed from solution by centrifugation. If extracts are centrifuged before activation occurs, then the stable, soluble proenzyme is obtained. Remixing the proenzyme and the lipid layer at the top of the centrifuge tube leads to activation. Bodine concluded that the natural activating factor was a lipid. A number of artificial activating agents were discovered (e.g. anionic detergents, organic solvents, urea, heat, heavy metals, and shaking). They all create denaturing conditions, and Bodine suggested that activation followed partial denaturation of the proenzyme. However, the possibility that a proteinaceous activating factor was denatured or not extracted is generally not considered in discussions of the Melanoplus enzyme. The weight of the evidence in most other insect systems strongly suggests this alternative (see below). Possibly, activation by denaturing agents is a purely artefactual phenomenon which bears no relation to the in vivo activation process. The active Melanoplus enzyme will oxidize both mono- and diphenols. It appears to contain copper since activity is lost on removal of copper and restored by cupric sulfate.

Tenebrio.

The phenol oxidase of Tenebrio molitor resembles that of Melanoplus (97, 98, 100). Centrifuged extracts of last instar larvae contain a stable proenzyme. The activator

is found not in the lipid layer but in the sediment. It is stable to heating at 100°C. Fractionation of lipids extracted from integuments led to the conclusion that eighteen carbon fatty acids function as phenol oxidase activators. No indications of a proteinaceous activator were found in whole extracts or in acetone powders. As in Melanoplus, negative evidence cannot be regarded as conclusive. The proenzyme was highly purified by DEAE-cellulose chromatography, column electrophoresis on Sephadex G-25, and sucrose gradient velocity centrifugation (99). The product was homogeneous on agar gel electrophoresis and in the analytical ultracentrifuge ($s_{20,w}$=7.3s). It contains 0.22% copper, 72-83% of which is in the reduced state in fresh preparations. After activation by sodium oleate, the enzyme will oxidize both mono- and diphenols.

Musca.

Musca phenol oxidase has not been examined in detail, but some information is available. A soluble proenzyme can be isolated from prepupae which lack the ability to convert it to active enzyme (103). An activator which appears to be a protein can be isolated at other stages. The proenzyme is also activated by treatment with SDS. The kinetics of activation by the protein activator give a normal Lineweaver-Burke plot suggesting that the activator acts catalytically on the proenzyme (104). Certain inhibitors

(N-bromosuccinimide, iodine, and sodium picryl sulfate) block the activation reaction. In crude extracts activation follows a sigmoidal time course (101, 102). The active enzyme forms very large aggregates.

Calliphora.

The phenol oxidase system of Calliphora erythrocephala has been studied in some detail by Karlson and coworkers. In fresh homogenates of larvae, no phenol oxidase activity is observed. After a concentration dependent lag period, activity increases in sigmoidal fashion (105). The shape of this curve resembles that expected for an autocatalytic process, and since an autocatalytic scheme had been proposed (see below), the kinetics of activation were analyzed at different concentrations. The data fit the theoretical autocatalytic curve only roughly. When the constant in the autocatalytic rate equation is evaluated, it is not constant at all but varies as a function of concentration. This result strongly argues against the autocatalytic mechanism. The activator, which is localized in the integument, and the proenzyme, which is localized in the hemolymph, have been separated and purified (106). When mixed, the purified components exhibit linear activation kinetics without a lag period. The possibility was suggested that the activator exists as an inactive precursor in vivo. Sedimentation constants of 2.9s and 15.4s are given for the activator and

proenzyme respectively. The criteria of purity presented seem inadequate, the purified components giving one band on analytical ultracentrifugation; it would seem unlikely that their preparations actually were homogeneous. The proenzyme can be activated by treatment with proteolytic enzymes (α-chymotrypsin and aminopeptidase) as well as the natural activator (107). This has been interpreted as suggesting activation by limited proteolysis although, actually, no data bearing directly on the normal activation mechanism have been presented. Curiously, a lag period is observed in the activation reaction catalyzed by proteolytic enzymes. The active enzyme has also been purified (108). Its purity may be questioned since it was analyzed only by paper electrophoresis and ultracentrifugation. The active enzyme is reported to have a sedimentation constant of 17s and a molecular weight of 530,000 daltons. It can oxidize only diphenols. When mixed with mitochondria, the enzyme absorbs to the mitochondria and acquires the ability to oxidize monophenols, presumably by coupling to some mitochondrial enzyme system (109).

The synthesis of the activator is apparently under control of the moulting hormone ecdysone (110). In permanent larvae produced by destruction of the ring gland, the activator concentration declines; injection of ecdysone leads to reappearance of the activator. Ecdysone has no effect on the proenzyme <u>in vitro</u> (111). On the basis of

this information, Karlson has proposed that, just prior to puparium formation, the activator diffuses into the cuticle and activates the latent phenol oxidase. This model of phenol oxidase activation *in vivo* may be questioned since there is no evidence that the cuticle is permeable to macromolecules. The possibility remains that active phenol oxidase is incorporated directly into the larval integument during epicuticle synthesis. According to this view, which is further discussed below, the phenol oxidase potential present at any given time is available for epicuticle synthesis and is not available for transport into preexisting cuticle.

Bombyx.

As in *Calliphora* and *Musca*, phenol oxidase activity appears in fresh homogenates of *Bombyx mori* with sigmoidal kinetics (112). The activator and proenzyme can be separated by ammonium sulfate fractionation; when remixed, activation is linear and without a lag period. Activation can be suppressed by high ionic strength. During the activation reaction, the active enzyme aggregates forming a polydisperse product of high molecular weight. The concentration of activator increases with time in the crude activation mixture, a phenomenon also observed in the Chinese oak silkmoth, *Antheraea pernyi* (113, 114). It has been proposed that the activator is derived from an inactive

precursor present in the initial homogenate.

Substantial information is available concerning the proenzyme (115). It has been purified from hemolymph by ammonium sulfate fractionation, heat treatment, DEAE-cellulose chromatography, and hydroxylapatite chromatography. The product, which is obtained in large quantities, is homogeneous on analytical polyacrylamide gel electrophoresis, SDS gel electrophoresis, and in the ultracentrifuge. The proenzyme contains 0.15 - 0.16% copper in the cupric state. Concentrated solutions are greenish blue with an absorption maximum at 650mμ. The molecular weight according to SDS gel electrophoresis is 80,000 daltons, but sedimentation equilibrium experiments indicate that this form is a dimer which can dissociate at low concentration. The sedimentation constant of the dimer is 6.6s. The isoelectric point of the proenzyme is 5.1 as determined by column electrofocusing. Noting that the copper in the proenzyme is in the cupric state and that the copper in active phenol oxidases including the Bombyx enzyme (117) is in the cuprous state, Ashida has proposed that the cupric copper is reduced during the activation reaction, probably by sulfhydryl groups in the proenzyme. The reduction of copper would then be a key event in the activation process. The proenzyme can be activated by proteolytic enzymes (α-chymotrypsin and the semialkaline protease of Aspergillus melleus) (116). This occurs without the aggregation usually seen in vitro. As

in Calliphora, the normal activation reaction is suspected to involve limited proteolysis, but no evidence bearing directly on this point has been adduced.

Drosophila.

Phenol oxidase has been known in Drosophila melanogaster since the report of Graubard in 1933 (118). The suitability of Drosophila for genetic analysis has lent considerable impetus to the study of Drosophila phenol oxidase. The activation reaction was described by Horowitz and Fling (119). As in several of the systems described above, activation is sigmoidal. The kinetics fit the theoretical curve for an autocatalytic process, and Horowitz proposed that activation involves conversion of the proenzyme into one molecule of active enzyme and one molecule of activator. Again following the established pattern, activation is accompanied by aggregation to a degree which permits removal of the active enzyme from solution by centrifugation. A number of metabolic inhibitors were tested on the activation reaction, but none blocked it. The active enzyme was poisoned by inhibitors of copper oxidases, suggesting that, as in all other cases studied, the Drosophila enzyme contains copper.

Mitchell investigated the activating system in detail, and his results define the problem under investigation in this thesis. Ammonium sulfate fractionation of extracts

prepared from pupae, yields fractions which produce no phenol oxidase activity separately but produce activity when mixed (120). When these fractions are subjected to electrophoresis on analytical polyacrylamide gels, no pigment bands appear when the gels are incubated in DOPA. If, however, the gels are preincubated in activator prepared from the 0-41% saturated ammonium sulfate fraction prior to incubation in substrate, intense melanin bands form. By this method, three components of the activating system may be distinguished. They have been named A_1, A_2, and A_3. Activation of the A_1 component yields an active enzyme which oxidizes both tyrosine and DOPA. When activated, A_2 and A_3 fail to oxidize tyrosine but are quite active on DOPA.

The tissue localization of the phenol oxidase components was studied in larvae and pupae by dissection methods (121). The result of this study was to localize one component of the activating system in the salivary gland. This component was named S (for salivary gland). It is thermolabile and behaves on fractionation as a protein. When mixed with the remaining components, designated collectively as H (for hemolymph), phenol oxidase activity appears with linear activation kinetics (122). Varying the concentration of S in an activation mixture affects the rate of activation but not the final phenol oxidase activity. S therefore appears to play a catalytic role in the activation process.

S itself has been shown to exist as an inactive precursor, pre-S, which can be isolated from four to seven hour prepupae in stable form (123). Activation of pre-S appears to be catalyzed by an enzyme, S-activator, and also by S. Thus, in fresh homogenates the S concentration is probably the rate limiting factor, and the kinetics observed by Horowitz and Fling probably reflect the activation of S which may be autocatalytic. As of this writing neither pre-S nor S-activator has been characterized. S, although difficult to handle, can be prepared in highly purified form (123). It has an approximate molecular weight of 35,000 daltons but has yet to be further analyzed. In a recent report, Drosophila S has been found to cross react with the Calliphora stygia proenzyme (124). The Calliphora equivalent of S was also found in the salivary gland. Due to the large size of the Calliphora salivary gland, it was possible to localize the activator more precisely than in Drosophila. It was not found in the secretory cells but only in the cuticular lining of the duct.

Mitchell's ammonium sulfate fractionation data suggested the existence of an additional component of the activating system called P. The existence of this component has recently been confirmed (123). At the present time the Drosophila phenol oxidase system appears to involve at least seven components, pre-S, S-activator, S, P, A_1, A_2, and A_3. Of course, structural interrelationships and further

heterogeneity of these components may be discovered.

Mitchell et al. prepared the active phenol oxidase from larvae and pupae of wild type and several mutant strains (125). When banded by equilibrium buoyant density centrifugation on sucrose gradients, each strain produced a characteristic pattern. A total of seven forms with distinct densities were observed. The sedimentation velocity of active enzyme was measured. The largest components sedimented at about one third the rate of Neurospora mitochondria, but no discrete size classes were observed. The active enzyme evidently varies continuously in size and discontinuously in density. A curious feature of the active enzyme is that it is inactivated at high centrifugal force (384,000xg). This effect is blocked by the addition of a small amount of substrate which leads to some melanization. The possibility was raised that covalent crosslinks form which stabilize the aggregate. On the basis of these properties, Mitchell suggested that phenol oxidase plays an unusual double role, being simultaneously structural and catalytic. The situation would then be somewhat analagous to the vertebrate melanosome.

Blumenthal examined the banded phenol oxidase from the ebony strain with a view that the structure of phenol oxidase is related to cuticle structure (126). He subjected this nearly intractable material, which has the appearance of a turbid, flocculent precipitate, to a number of violent de-

naturing conditions and strong solvents. The aggregation could be reversed only partially; at least 25% of every preparation appeared to be covalently crosslinked. When analyzed by SDS gel electrophoresis, the solubilized protein did not produce any consistent pattern from preparation to preparation, and it proved difficult to construct a model for the structure of the aggregate.

Mitchell et al. studied the developmental time course of phenol oxidase potential in wild type and four mutant strains (ebony, black, blond, and yellow) (125). All strains exhibited points of maximal potential activity just before puparium formation and in the range of 55 to 70 hours after puparium formation. Although the same general pattern was observed in all cases, each strain studied had a characteristic time course. These results were interpreted in terms of altered regulation of phenol oxidase activity rather than mutations in the phenol oxidase structural genes. Other genetic studies have been carried out by Lewis and Lewis (127, 128). They were able to select for strains with low and high levels of phenol oxidase. The genetic variability of phenol oxidase activity was considered to reflect the function of several modifier genes. A strain with low activity, a, behaved in a manner suggesting that the a locus directly affects phenol oxidase structure. The phenol oxidase produced by homozygeous a adults is more thermolabile than the wild type enzyme, and it has an altered substrate speci-

ficity. *a* maps at 52.4 on chromosome II and may be a structural gene for one of the phenol oxidase components. In light of current knowledge, this conclusion should be regarded as quite tentative since alteration in the relative proportion of the various components might account for Lewis' results. A claim has been made that the A_1 component is absent in strains carrying mutant alleles at the *lozenge* locus (129). This claim does not seem fully justified by the data presented which include only analytical polyacrylamide gels of small amounts of homogenate.

Recently, Mitchell *et al.* have studied the process of cuticle deposition in larvae by electron microscopy and autoradiography (130). The most important result of this investigation relevant to the phenol oxidase problem is the absence of any pores or canals in the chitinous layer of larval cuticle. It appears unlikely that any macromolecular material could diffuse through the cuticle. This conclusion is particularly clear cut in the case of the greatly aggregated active phenol oxidase. Autoradiographic experiments support this view. Injected tritiated leucine produces a band of label in the white puparial cuticle which marks the position of the cuticle cell interface at the time of injection. Low molecular weight molecules evidently can diffuse outward since injected tritiated tyrosine strongly labels the epicuticle during puparium formation. Mitchell advanced the proposition that the major proteins of the

cuticle are incorporated in a fixed position at the time of cuticle synthesis; they may be modified subsequently by low molecular weight substances. As Mitchell has pointed out, the diffusion of an activator enzyme into the cuticle cannot be ruled out. A catalytic activator might be required in only very small quantities. The conclusion does seem inescapable that the large phenol oxidase potential present at puparium formation is not related to the hardening and darkening of the puparium.

Yamazaki has attempted to prepare a cuticular phenol oxidase directly from acetone powders of Drosophila virilis integuments (131). Only small amounts of activity were extracted which may represent contamination by the abundant hemolymph enzyme. Yamazaki argued that his preparations had the substrate specificity of a laccase. However, his data show some oxidase activity on tyrosine. From what is known of the active hemolymph enzyme, it is unlikely that it could be extracted from cuticle in anything resembling its native form. Hackman and Goldberg have prepared extracts from the cuticles of several insects which oxidize tyrosine and DOPA (132).

At this point, it should be noted that, although the cuticular functions of phenol oxidase have been emphasized, there is little direct evidence connecting the hemolymph enzyme with cuticle synthesis. Other related functions have been proposed. Specifically, phenol oxidase is implicated in

wound healing. Lai-Fook has demonstrated the presence of a latent phenol oxidase in the cuticle of several insects which can be activated by abrasion (133). He drew attention to the difficulty of distinguishing this enzyme from the oxidases responsible for normal cuticular hardening and darkening. Some of the conflicting reports in the insect phenol oxidase literature may well be due to this problem. An interesting case of wound healing by phenol oxidase was noted in locust eggs (134). Several authors have also observed the role which the Drosophila hemolymph phenol oxidase plays in the defense against invading parasites (135, 136, 137, 138). In Drosophila melanogaster, eggs of Pseudocoila mellipes are enveloped by crystal cells, which are known to contain phenol oxidase (139), and sealed into a melanized capsule. This effectively blocks further development of the parasite.

Research Outline.

At the beginning of the research reported in this thesis, it was very clear that further elucidation of the structure and mode of activation of Drosophila phenol oxidase, its genetic and developmental control, as well as its in vivo function depended on the isolation, purification, and characterization of the protein components of the system. The active enzyme had yielded little information, and although the basic components of the activation system had been

identified, it was impossible to draw any firm conclusion concerning their roles in the activation process. It was not even clear, for example, which combinations of components could produce active enzyme. It was equally uncertain which components were incorporated into the final product. The fractions containing the various components were either unstable or too cross-contaminated to permit simple definitive experiments. Therefore a program of purification of the components involved in the activating system was initiated. This thesis will be concerned primarily with the purification and properties of the A components. Work on the activating factors pre-S, S-activator, S, and P will be reported elsewhere.

MATERIALS AND METHODS

1. Drosophila Culture.

All stocks were obtained from the stock center at the California Institute of Technology. Drosophila melanogaster Oregon R eggs were collected and cultured according to Mitchell and Mitchell (141). Late third instar larvae were collected on the fifth day after egg laying when at least 10% of the larvae had formed puparia. The washed, dried larvae were frozen in liquid nitrogen and stored at -70°C. Carefully staged 4 to 7 hour prepupae, used in some experiments, were collected as follows. This procedure takes advantage of the fact that an air bubble is secreted 4 hours after puparium formation. Larvae and prepupae were washed into a two liter beaker which was filled to capacity with tap water. Any animals which floated to the top of the beaker were removed by decanting and discarded. This process was repeated until no more animals were observed to float. The beaker was then covered with cheesecloth, drained and incubated at 25°C. for three hours. The floating and decanting process was then carried out. Floating animals were saved, re-floated and re-decanted in a separate beaker. The second stage of floating and decanting removed any non-floating animals which might have adhered to floating animals. The animals thus selected were then washed, dried, and inspected by eye for dead, malformed, or incorrectly staged

animals. Any such animals were removed with watchmaker's forceps. The acceptable animals were frozen in liquid nitrogen and stored a -70°C. The 2 liter beaker was then incubated for another 3 hours, and the entire process was repeated until the bulk of the larvae in the culture had formed puparia. Great care must be exercised in the selection of 4 to 7 hour prepupae or their homogenates will produce phenol oxidase activity.

2. Preparation of Crude S.

Two methods of preparation are given. The first was used for Assay I (see section 4A. below); the second was used for Assay II (see section 4B. below).

A. Method I.

Fifty grams of late third instar larvae were ground to a fine powder with 25g of sand in a mortar chilled with liquid nitrogen. The powder was added with stirring to 750ml of 1.0M urea in 0.1M phosphate buffer, pH 6.3 at 20°C. All subsequent handling was performed at 4°C. The extract was filtered through a double thickness of cheesecloth and centrifuged at 13,000 r.p.m. for 15 minutes in the GSA rotor of the Servall RC2-B centrifuge. The supernatant was filtered through nylon cloth to remove the fatty layer. Cold saturated $(NH_4)_2SO_4$ was added slowly with stirring to 41% saturation. The precipitate was pelleted by centrifugation

at 9,000 r.p.m. for 50 minutes in the GSA rotor. The pellet was resuspended in 50ml of 0.1M TrisHCl, pH 8.5 and dialyzed for 5 hours against 500ml of 0.1M phosphate buffer, pH 6.3 with one 500ml change after 2.5 hours. The dialyzed solution was centrifuged at 25,000 r.p.m. for 30 minutes in the Type 30 rotor of the Beckman Model L ultracentrifuge. The supernatant was divided into 0.5ml aliquots and frozen at -70°C.

B. Method II.*

The following procedure was carried out at 4°C. Ten grams of late third instar larvae were ground to a smooth paste in a mortar and pestle with 10g of sand and 15ml of 35% saturated $(NH_4)_2SO_4$. The homogenate was diluted with 135ml of 35% saturated $(NH_4)_2SO_4$ and centrifuged at 25,000 r.p.m. for 15 minutes in the Type 30 rotor of the Beckman Model L ultracentrifuge. The pellet was resuspended in 100ml of 35% saturated $(NH_4)_2SO_4$ and centrifuged for 10 minutes at 25,000 r.p.m. in the Type 30 rotor. The pellets were washed once more in the same fashion. The pellet was then resuspended in 100ml of 0.1M phosphate buffer, pH 6.3, and the solution was brought to 35% $(NH_4)_2SO_4$ saturation. The precipitate was collected by centrifugation at 25,000 r.p.m. for 15 minutes in the Type 30 rotor. The pellet was

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* This procedure is based on suggestions from Mr. D. Seybold.

then resuspended in 5ml of 0.1M phosphate, pH 6.3. The solution was dialyzed against 500ml of 0.1M phosphate, pH 6.3 for 3 hours and cleared by centrifugation at 25,000 r.p.m for 10 minutes. The supernatant was divided into 0.2ml aliquots and frozen at -70°C.

3. Phenol Oxidase Assay.

Phenol oxidase activity was assayed as described by Mitchell (142). In this assay, one unit of phenol oxidase activity is defined as that amount for which the $\Delta O.D._{475m\mu}$ per 10 minutes equals 1.0. Blumenthal has shown that the phenol oxidase assay responds to enzyme concentration in a linear fashion (126).

4. A Component Assay.

Two methods were used for the quantitative assay of the A components. The first was superceded by the second.

A. Assay I.

A 20µl aliquot of the unknown was incubated at 4°C. for 30 minutes with 20µl of crude S (Method I, see section 2A. above) and 20µl of H.* The mixture was then assayed in the phenol oxidase assay.

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*The H fraction was prepared from late third instar larvae by Dr. D. Sullivan. H stands for hemolymph; it is the crude 37.5 to 56% saturation $(NH_4)_2SO_4$ fraction.

B. Assay II.

A 20µl aliquot of the unknown was incubated at 4°C. for 30 minutes with 20µl of crude S (Method II, see section 2B. above). The mixture was then assayed in the phenol oxidase assay. One unit of A component activity is defined as that amount which produces one unit of phenol oxidase activity in this assay. The standard curve for this assay is discussed below.

5. Preparation of Crude A Components.

Two procedures are given for the preparation of crude A components. The first uses late third instar larvae, the second uses 4 to 7 hour prepupae.

A. Preparation of A Components From Larvae.

One hundred grams of late third instar larvae were ground to a fine powder with 100g of sand in a mortar chilled with liquid nitrogen. The powder was added with stirring to 1.0 l of 1M urea in 0.1M phosphate, pH 6.3 at 20°C. The following steps were carried out at 4°C. The homogenate was filtered through a double thickness of cheesecloth and centrifuged at 9,000 r.p.m. for 10 minutes in the GSA rotor of the Servall RC2-B centrifuge. The supernatant was filtered through a double thickness of cheesecloth and slowly brought to 37.5% $(NH_4)_2SO_4$ saturation with saturated $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 9,000 r.p.m. for

40 minutes in the GSA rotor. The supernatant was filtered through nylon cloth and brought to 56% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 9,000 r.p.m. for 40 minutes in the GSA rotor. The pellet was then washed by resuspending it in 50ml of 56% saturated $(NH_4)_2SO_4$ and centrifuging at 25,000 r.p.m. for 15 minutes in the Type 30 rotor of the Beckman L-2 ultracentrifuge. (Pellets were sometimes stored at this stage by freezing at -70°C.). The pellet was then resuspended in 7ml of 1.5M KCl in 0.1M TrisHCl, pH 8.5 and centrifuged for 15 minutes at 25,000 r.p.m. in the Type 30 rotor. The supernatant was saved, and the pellet was suspended once more in 6ml of the same buffer and centrifuged as before. The second supernatant was combined with the first for further processing. The combined supernatants were then subjected to heat treatment (see section 6. below).

B. Preparation of Crude A Components From Prepupae.

This procedure was carried out at 4°C. Twenty grams of 4 to 7 hour prepupae were rapidly ground to a smooth paste in a mortar and pestle with 20g of sand and 40ml of 37.5% saturated $(NH_4)_2SO_4$. The paste was diluted with 260ml of 37.5% saturated $(NH_4)_2SO_4$ and centrifuged at 25,000 r.p.m. for 15 minutes in the Type 30 rotor of the Beckman Model L ultracentrifuge. The supernatant was filtered through nylon cloth and brought slowly to 56% $(NH_4)_2SO_4$ saturation by ad-

dition of saturated $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation at 25,000 r.p.m. for 15 minutes in the Type 30 rotor. The pellet was washed by resuspending it in 40ml of 56% saturated $(NH_4)_2SO_4$ and centrifuging as in the previous step. The pellet was then resuspended in 10ml of 0.1M phosphate, pH 6.3. The solution was cleared by centrifugation at 25,000 r.p.m. for 15 minutes in the Type 30 rotor.

6. Heat Treatment.

Some crude A component samples were subjected to heat treatment by incubation in a 40°C. water bath for 30 minutes. Heating was terminated by plunging the sample tube into ice water. Any precipitated protein was removed by centrifugation at 25,000 r.p.m for 15 minutes in the Type 30 rotor.

7. Gel Filtration.

Sephadex (G-25 fine, G-100 medium, and G-200 medium, Pharmacia Fine Chemicals) was hydrated in distilled water and washed several times by settling in distilled water. The gel was then equilibrated in elution buffer. Columns were poured at exactly the elution pressure to be used. Void volumes were occasionally measured with Blue Dextran (Pharmacia Fine Chemicals). All gel filtration experiments were carried out at 4°C.

8. Preparative Acrylamide Gel Electrophoresis of A.

The Canalco Prep-Disc apparatus (Canal Industrial Corp.) was used for preparative polyacrylamide gel electrophoresis. A 1.5cm 10% acrylamide separating gel was used with a 2.5cm spacer gel. Gels were prepared according to the Canalco formulas. The medium column (PD2/150) was used for most experiments. The upper electrode buffer contained glycine (2.88g/l) and Tris (0.6g/l). The lower electrode and elution buffer was 0.03M TrisHCl, pH 8.5. The A_1 sample, previously dialyzed against one quarter strength upper electrode buffer, was layered onto the spacer gel in 10% sucrose; a drop of 0.005% bromphenol blue was added to the sample before layering. The elution buffer was pumped through the column at a rate of 1ml/min. The anode was placed at the bottom of the column. Electrophoresis was started with a constant current of 9ma for 2.25 hours. The current was then increased to 12ma for 4 hours. The elution buffer was collected in 5ml fractions. Fractions were assayed for A component activity.

9. Preparative Isoelectric Focusing.

Two methods are given for the preparative isoelectric focusing of the A components. The first uses a 2 pH unit gradient for A_1 and A_2. The second is a special expanded, preformed gradient for A_1.

A. Two pH Unit Gradient.

Preparative isoelectric focusing was carried out in

the LKB 8101 column. The 8102 column was used in some experiments using a direct scale-up of the following method. A stabilizing sucrose gradient was poured exactly as recommended by the LKB instruction manual. A 1% ampholyte solution (pH 4 to 6 range) was used. The sample, after dialysis for five hours against a 0.5% ampholyte solution, was introduced into the less dense sucrose gradient mixing chamber. The anode was placed at the bottom of the column. Tap water was circulated through the cooling jacket. Focusing was initiated by applying a potential sufficient to draw 3 watts. The power drawn by the column was maintained at this level by increasing the applied voltage until 1000v was reached. The voltage was maintained at 1000v until the experiment was terminated at 48 hours after first applying voltage. The column was then drained at a rate of 2ml/min. 3ml fractions were collected. The pH and optical density at 280mμ of the fractions were determined. They were then assayed for A component activity.

B. Expanded, Preformed Gradient.

The LKB 8101 column was used for these experiments. The first step consists of fractionating the ampholytes. This was done by setting up a standard focusing experiment as described above. Instead of the normal ampholyte concentration an 8% solution of pH 5 to 7 ampholytes was used without any protein sample. The column was focused to equi-

librium (total focusing time 72 hours, maximum voltage 600v). Two ml fractions were then collected, and the pH of each fraction was determined. The fractions spanning the range pH 4.5 to pH 6.0 (23 fractions) were frozen at -20°C. until further use. For an actual protein fractionation experiment, the stabilizing sucrose gradient was introduced in 23 steps. These were divided into 3 parts. The first part was prepared by pouring a 28ml gradient into seven 4ml fractions (heavy solution: 14ml of 60% sucrose + 100µl of 40% pH 3 to 10 ampholyte solution; light solution: 14ml of 46% sucrose). The second part consisted of 5ml of carefully desalted protein sample in 43% sucrose. The third part was a 60ml sucrose gradient poured into 15 4ml fractions (heavy solution: 30ml of 39% sucrose + 100µl of 40% pH 3 to 10 ampholytes; light solution: 30ml of distilled water). Thus a total of 23 tubes were prepared. To each of these was added 0.5ml from the corresponding tube of the fractionated ampholytes prepared above. The resulting series of tubes then contained a stepwise sucrose gradient and a pH gradient. The gradient was then pumped into the column at the rate of 4ml/min. The column was chilled to 9°C., a potential of 800v was then applied to the column. After two hours, the voltage was increased to 1000 volts. The power was turned off after 12 hours, and the column was drained at the rate of 2ml/min., and 2ml fractions were processed as in part 9A. above.

10. DEAE-Sephadex Chromatography of A_2.

DEAE-Sephadex A-25 (Pharmacia Fine Chemicals) was hydrated in distilled water and washed several times by settling in distilled water. The pH of the slurry was adjusted to the desired value with 1.0M Tris base. The gel was then washed several times with the elution buffer (0.05M Tris-HCl, pH 6.8) by settling. A_2 does not absorb to DEAE-Sephadex under these conditions. Samples of A_2 equilibrated with the elution buffer by dialysis were passed over a 0.5ml bed of this gel in a Pasteur pipette at 20°C.

11. Hydroxylapatite Chromatography of A_2.

Biogel HT (Bio-Rad Laboratories) was washed several times by settling in 1mM sodium pyrophosphate, pH 6.8. The slurry was poured into a Pasteur pipette until a 0.25ml bed formed. The following steps were carried out at 20°C. The column was washed with 1mM pyrophosphate buffer under pressure produced by a rubber bulb. The A_2 sample in 0.05M Tris-HCl, pH 6.8 was then applied to the column. The column was washed with sodium pyrophosphate, pH 6.8 in the following concentrations and quantities: 1mM, 0.5ml; 0.2M, 0.5ml; and 0.3M, 1.5ml. Fractions (0.25ml) were collected and assayed for A component activity.

12. Concentration Methods for the A Components.

A. $(NH_4)_2SO_4$ precipitation.

Samples containing at least 1mg/ml of protein were concentrated by the addition of cold, saturated $(NH_4)_2SO_4$ to 60% saturation. The precipitate was then collected by centrifugation at 4°C.

B. Ultrafiltration.

Samples containing less than 1mg/ml of protein were frequently concentrated with the Amicon ultrafiltration apparatus. The PM-10 membrane was used exclusively; no problems due to adsorption to the membrane were encountered. Occasionally, samples were desalted by repetitive addition of distilled water to the pressure dialysis cell.

13. Analytical Polyacrylamide Gel Electrophoresis.

Analytical electrophoresis in polyacrylamide gels was carried out by the standard method of Davis (143). Staining with amido black was done as Davis describes. In some experiments gels were stained in a 0.25% solution of Coomasie Brilliant Blue in 7% acetic acid. Gels were destained by diffusion in 7% acetic acid. Gels to be developed for A component activity were processed according to Mitchell and Weber (120) with the slight modification that crude S, prepared as described above (see section 2), was used as the activator.

14. Method for Testing Mutant Strains for Electrophoretic Variants of the A Component.

This procedure was carried out at 4°C. Forty late third instar larvae (wild type or mutant) were rapidly homogenized in a glass cone homogenizer containing 2.0ml of homogenizing buffer (1.0M urea, 2.88% glycine, 0.6% Tris, 1% disodium EDTA, and 0.00025% bromphenol blue). The homogenate was centrifuged for 5 minutes at 30,000 r.p.m. in the Type 40 rotor of the Beckman Model L ultracentrifuge. Two aliquots of the supernatant were analyzed for A component activity in the electrophoretic assay (see section 13 above). The first aliquot of 100µl was analyzed directly without further processing. The second aliquot of 50µl was mixed with 50µl of a similarly prepared extract of wild type larvae. The mixture was then analyzed for A component activity. The processed analytical gel of the first sample was used to verify that detectable quantities of the A components were present. The processed analytical gel of the second sample was inspected for the presence of A component with unusual electrophoretic mobility.

15. SDS Polyacrylamide Gel Electrophoresis.

SDS polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (144). Cytochrome c, myoglobin, chymotrypsinogen, ovalbumin, bovine serum albumin, glutamic acid dehydrogenase, phosphorylase a, and catalase were used as molecular weight standards. Their

mobilities relative to bromphenol blue were calculated and plotted as described by Weber and Osborn. A linear standard curve was obtained. In addition to the standard sample application buffer described by Weber and Osborn (0.01M phosphate, pH 7.0, containing 0.1% SDS and 0.1% β-mercaptoethanol), the following sample application buffers were occasionally used: same as the standard buffer but containing 8M urea or 1% SDS. In addition to the Weber and Osborn technique of incubation at 37°C. for 2 hours before electrophoresis, samples were sometimes heated at 50°C. for one hour, 86°C. for one hour or 100°C. for 15 minutes in the buffers described above.

16. Analytical Electrofocusing.

Analytical electrofocusing in polyacrylamide gels was carried out as described by Wrigley (145). Since the A_1 component was found to be unstable under the conditions of the gel polymerization reaction, the layering method of sample application was used.

17. Analytical Ultracentrifugation.

Band sedimentation experiments were carried out in the Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. The Type ANA rotor was used with a double sector cell. The sedimentation medium was 90% D_2O, 0.1M NaCl in 0.01M TrisHCl, pH 7.9. The temperature was

20°C. The rotor speed was 52,000 r.p.m., and $O.D._{280}$ recordings were made at 8 minute intervals. Physical constants for D_2O were taken from the report of Baker and La Mer (149).

18. Lipid Determination.

Samples of purified A_1 prepared according to Table 3 (100µl, containing 25µg of protein in 0.01M TrisHCl, pH 7.2) were mixed with an equal volume of 4M KOH in a sealed tube and heated in a boiling water bath for 2 hours. The hydrolysates were cooled, diluted with 0.5ml of H_2O and acidified with 15µl of concentrated H_2SO_4. The hydrolysate was then extracted 3 times with 0.5ml of chloroform. The extracts were pooled and made up to 5.0ml with chloroform. The extracts were then analyzed for free fatty acids by the extremely sensitive method of Mahadevan et al. (146) which can detect as little as 10 nanomoles of fatty acid. This procedure was followed exactly as described with the slight modification that the extracts were not diluted for assay as Mahadevan et al. describe. Buffer blanks processed as described above gave values equal to those obtained with pure chloroform. A series of lauric acid standards gave a linear standard cure (see below).

19. Phosphate Determination.

Phosphate was determined by the method of Chaldvarjian and Rudnicki (147) which can detect as little as 1.0 nano-

mole of phosphate. 100µl samples of purified A_1 prepared according to Table 3 were analyzed (250µg/ml of protein in 0.01M TrisHCl, pH 7.2). Samples of BSA at the same level of concentration gave analyses equivalent to the H_2O blank. Phosphate standards gave a linear standard curve (see below).

20. Protein Determination.

Protein was determined by the method of Lowry (147).

21. Copper Determination.

Total copper content was determined by the oxalyl dihydrazide method of Stark and Dawson (148), except that 0.5M HCl was used to release the copper, and the final reaction volume was reduced to 0.5ml. Active phenol oxidase (ebony heavy band 1.70mg/ml) was prepared by Blumenthal (126). 300µl aliquots of the active enzyme were analyzed. 150µl aliquots of purified A_1 prepared by the method outlined in Table 3 (0.63mg/ml) were analyzed. Copper standards gave a linear standard curve (see below).

22. Amino Acid Analysis.

Protein samples were hydrolyzed in 6N HCl in sealed vials at 110°C. for 12 hours. The amino acid content of the hydrolysate was analyzed on a Beckman Amino Acid Analyzer. This analysis was carried out through the courtesy of Dr. John Smart.

23. Reagents.

Urea was Mann Ultra Pure (Mann Research Lab.). Acrylamide and methylenebisacrylamide were Canalco Prep-Disc grade. SDS was technical grade (Matheson, Coleman, and Bell). DOPA was from Nutritional Biochemical Co. Tris was Trizma Base from Sigma Chemical Co. Myoglobin was Calbiochem A grade. Bovine serum albumin, catalase, ovalbumin, glutamic acid dehydrogenase, phosphorylase a, and chymotrypsinogen were from Sigma Chemical Co.

Saturated $(NH_4)_2SO_4$ was prepared in 0.1M phosphate buffer, pH 6.3 without readjustment of the final pH, and partially saturated solutions were always prepared by dilution with 0.1M phosphate buffer, pH 6.3.

RESULTS

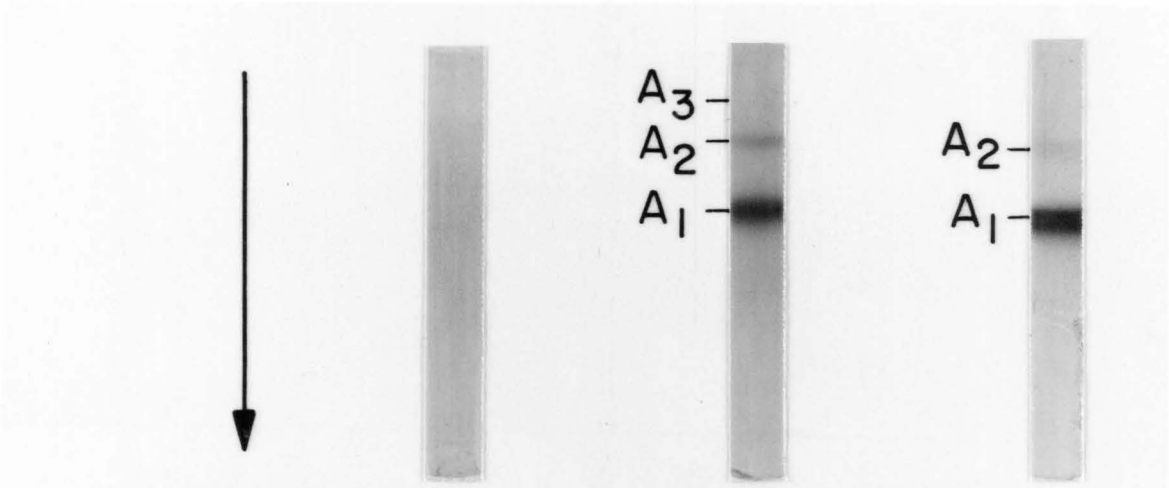
1A. Electrophoretic Assay of A Components.

The A components of Drosophila phenol oxidase are identified by reference to their electrophoretic mobility on polyacrylamide gels. Figure 1 shows a series of such gels. If the gels are not preincubated in crude S, no melanin is deposited during incubation in substrate. The A_2 and A_3 components have nearly the same electrophoretic mobility and are not always readily separated by this technique. The A_1 component is distinguished by its substrate specificity since it is the only component which gives rise to an enzyme which can act on monophenols. The electrophoretic assay was used routinely to determine the A component composition of samples containing latent phenol oxidase activity.

1B. Survey of Mutant Strains for Electrophoretic Variants of the A Components.

The electrophoretic assay described above was used to screen a series of strains carrying mutations affecting cuticular structures for electrophoretic variants of the A components. Late third instar larvae were extracted, and the extracts analyzed as described in Materials and Methods (section 14). The following strains were tested: tan-1 (t^1)., ebony-11 (e^{11}), black (b), yellow (y), Blond (T(1;2)Bld/Clb), alpha (a b cn and a p^p). No electrophoretic variants of the A components were detected in these strains.

Figure 1. The electrophoretic assay of the A components. An extract of 4 to 7 hour prepupae was prepared according to Materials and Methods (section 14). Aliquots containing an amount of extract corresponding to 1.5 animals were subjected to electrophoresis on polyacrylamide gels (see Materials and Methods, section 13). Gel number 1 was incubated in substrate without activation in crude S. Gels 2 and 3 were incubated in substrate after treatment in crude S. The faint A_3 band in the original gel is not reproduced in the photograph. The arrow at the left indicates the direction of migration.



Sample:	Crude A	Crude A	Crude A
Substrate:	DOPA	DOPA	TYR
Activation:	None	Crude S	Crude S
Gel Number:	1	2	3

2. Quantitative Assay of A Components.

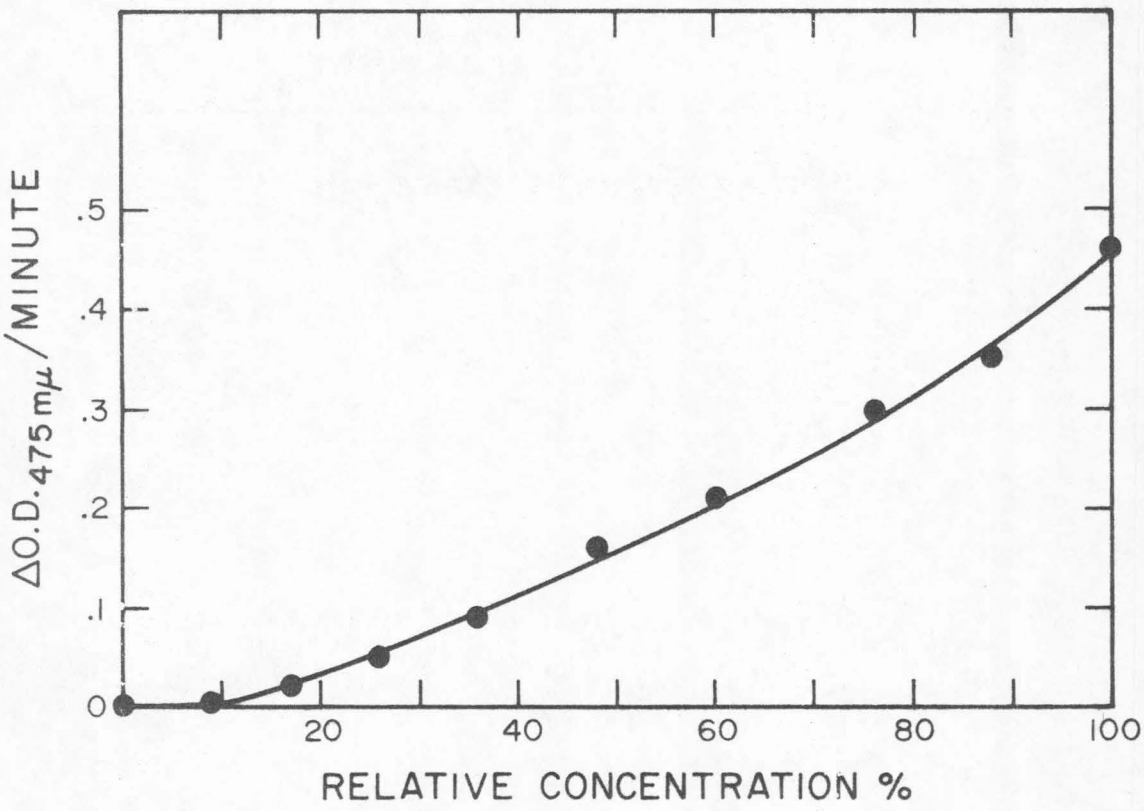
Development of handling methods and purification procedures for the A components depended upon the availability of a quantitative assay for latent phenol oxidase activity. Two such methods were employed in this study (see Materials and Methods, section 4.), the first was superceded by the second. In the first method (Assay I), the unknown is incubated for a standard time with equal volumes of crude S and H. The mixture is then assayed for phenol oxidase activity. The H was included in the assay mixture because crude S preparations were found to vary in their ability to activate purified A components. For example a crude S preparation which could activate crude A components might have no activity with purified components. Another sample of crude S, prepared by the same method, might activate both pure and crude A components. It appeared that there was an essential activating factor with an ammonium sulfate precipitation profile which overlaps those of S and the A components and which was removed from the crude A components during purification. This factor could be supplied by adding H to the assay mixture. Later, a more reliable method was developed for preparing crude S which always could activate purified A components; such crude S presumably contains both activating factors, S and P. This is the foundation of the second assay method (Assay II). In this assay, an aliquot containing the unknown quantity of A components is incubated with an equal

volume of crude S; the phenol oxidase activity is assayed after a fixed incubation period. The standard curve for this assay is given in Figure 2. This curve deviates strongly from a straight line. In order to obtain reliable quantification of A component activities, samples were concentrated or diluted as necessary to bring them into the 1 to 2 unit range. In this range, which is a convenient working level of activity, the assay curve can be approximated by a straight line. Another advantage of working in this range is that the crude S concentration is not a critical variable since doubling the incubation time does not alter the level of activity obtained. The assay curve demonstrates an important difficulty encountered in working with the A components. The activation reaction is extremely sensitive to dilution. Ten fold dilution of a sample which can readily be assayed may well bring it out of the useful range of the assay. For this reason, it is necessary to begin fractionation procedures which involve dilution with very concentrated solutions of A components.

3. Blocking the Activation Reaction.

The tendency of Drosophila homogenates to convert the A components to active phenol oxidase was the major obstacle to be surmounted in the purification of the A components. If the activation reaction is not blocked at all stages in the purification, particularly during the early stages when relatively large quantities of S and P may be present, complete activation may occur in a matter of minutes. Even

Figure 2. The standard curve for Assay II. Crude A was prepared according to Materials and Methods (section 5B) from 4 to 7 hour prepupae. The extract was dialyzed against 0.01 M TrisHCl overnight, and a series of dilutions of the dialyzed solution was prepared and assayed for A component activity in Assay II (see Materials and Methods, section 4B). The abscissa indicates the dilution of the sample assayed for each point relative to the undiluted dialyzed extract. Thus 100% refers to the undiluted extract, and 50% refers to the undiluted extract mixed with an equal volume of buffer.


ΔO.D.475mμ/MINUTE
0
.1
.2
.3
.4
.5
20
40
60
80
100
RELATIVE CONCENTRATION %

partial activation is decidedly undesirable since small amounts of phenol oxidase activity will lead to melanization and loss of A component activity. Many preparations were lost to activation before reliable procedures were developed. Fortunately, several methods are available to block the activation reaction. Used singly and in combination, they permit relatively facile handling of the A components. The first of these methods is the use of high ionic strength. Buffers containing 1.5 M KCl were found to block activation completely. This method suffers from the disadvantage that the salt must be removed or diluted out before the A component assay can be performed. Of course, many fractionation procedures cannot be carried out in the presence of high ionic strength buffers. A second method of blocking activation is the use of 1 M urea. Although this concentration of urea does not block activation completely, it approximately doubles the lag period in the activation of crude homogenates. It is therefore useful in the preparation of crude extracts. The simplest method for blocking activation is dilution. Activation in very dilute solutions is greatly retarded; this is part of the rationale behind the large volume of extraction medium used in the initial stages of purification. The A components differ in thermostability from the activating factors. This is illustrated in Table 1. Heating at 40°C. has no measurable effect on the A component activity, but appreciable crude S activity is lost at this temperature.

Table 1.

The Stability of Crude A and Crude S to Heating at 40°C.

Sample	Time at 40°C. (minutes)	% Initial Activity
Crude S	0	100
	5	63
	10	46
	15	38
Crude A	0	100
	5	100
	10	100
	15	100

Crude A was prepared according to Materials and Methods (section 5A). A component activity was assayed in Assay I (see Materials and Methods, section 4A). Crude S was prepared according to Materials and Methods (section 2A) and assayed for S activity by testing its activity on crude A in Assay I.

Thus, extracts can be protected from activation by heating at 40°C.

The most effective method for blocking activation is utilization of 4 to 7 hour prepupae as starting material. At this stage, no phenol oxidase activity is produced by homogenates, even after prolonged incubation (125). However, the A assay reveals large quantities of A components. Thus, precisely staged prepupae can be used as a source of stable A components. This approach is not without difficulty. Staging prepupae is a tedious and time consuming task. The yield of staged animals is reduced substantially by comparison to unstaged cultures. However, the low yield is compensated for by the consistently high A component content of staged animals. The peak phenol oxidase potential is normally observed just before puparium formation (125). Cultures of late third instar larvae, especially when crowded, may contain immature larvae which have not yet reached the peak phenol oxidase potential. Selection of prepupae provides assurance that the cultures are mature and healthy.

4. Preparation of Crude Extracts.

Blocking activation as outlined above, two types of crude extracts have been prepared. In the first type, late third instar larvae are ground to a fine powder in liquid nitrogen and extracted in buffer containing 1.0 M urea. The 37.5% to 56% saturated $(NH_4)_2SO_4$ fraction is prepared and

heat treated at 40°C. in buffer containing 1.5 M KCl. Subsequent handling would then use high ionic strength buffer whenever possible. In the second procedure, 4 to 7 hour prepupae are ground in 37.5% saturated $(NH_4)_2SO_4$, a condition which extracts A_1 preferentially (see below). The 37.5% to 56% saturated $(NH_4)_2SO_4$ fraction is then prepared. In contrast to larval extracts, prepupal extracts have little tendency to activate even on prolonged incubation (24 hours) at 4°C. An average of 300 units of A component activity per gram of animals was obtained. This represents a substantial improvement over the highly variable figure of 50 to 150 units per gram obtained with larvae.

5. Fractionation Procedures.

A. General.

The lability of the A components under conditions which permit activation places severe restrictions on fractionation methods. Adsorption techniques such as ion exchange chromatography were found to be generally inapplicable. Adsorption to a column of any sort (DEAE-cellulose, DEAE-Sephadex, SE-Sephadex, hydroxylapatite, and Celite 535) was always associated with complete or partial activation and consequent poor recovery. The concentration of A component activity could never exceed 1000 units per ml. except in the presence of 1.5 M KCl or in very pure preparations. Generally, concentrations higher than 500 units per ml. were

avoided since trace quantities of the activating factors might be concentrated to levels sufficient to bring about activation.

B. Gel Filtration.

The behavior of crude A components on Sephadex G-200 and G-100 is shown in Figures 3 and 4. Only a single peak of A component activity was observed in 27 experiments. The K_{av} values of 0.46 and 0.18 for G-200 and G-100 respectively correspond to a molecular weight of 77,000 daltons (151). Recovery of A component activity from gel filtration columns was 80%. Since the bulk of the protein present in crude extracts can be separated from the A components by gel filtration, this constitutes a useful technique. A 6 to 9 fold purification is obtained on G-200 chromatography of crude extracts.

C. Preparative Isoelectric Focusing.

Preparative isoelectric focusing in pH gradients of synthetic ampholytes is of great utility in the purification of the A components. The behavior of the A components on electrofocusing is shown in Figure 5. In this experiment, which used larval material, two peaks of A component activity were obtained, one peak at pH 6.0 and another at pH 5.1. The pH 5.1 peak shows some sign of splitting into two peaks. This has appeared reproducibly in several experiments. When

Figure 3. G-200 chromatography of crude A. The sample was crude A prepared from 20 g of 4 to 7 hour prepupae. The column was 4.0 X 67 cm. The elution buffer was 0.02 M TrisHCl, pH 7.2. The flow rate was 0.46 ml/min. Fractions were collected at 20 minute intervals. 20 µl aliquots of each fraction were assayed in Assay II. The elevated baseline in the A component elution profile (closed circles) is due to a small amount of phenol oxidase activity (0.5 u per 20 µl) in the crude S used in the assay and is not due to spreading of the A activity peak.

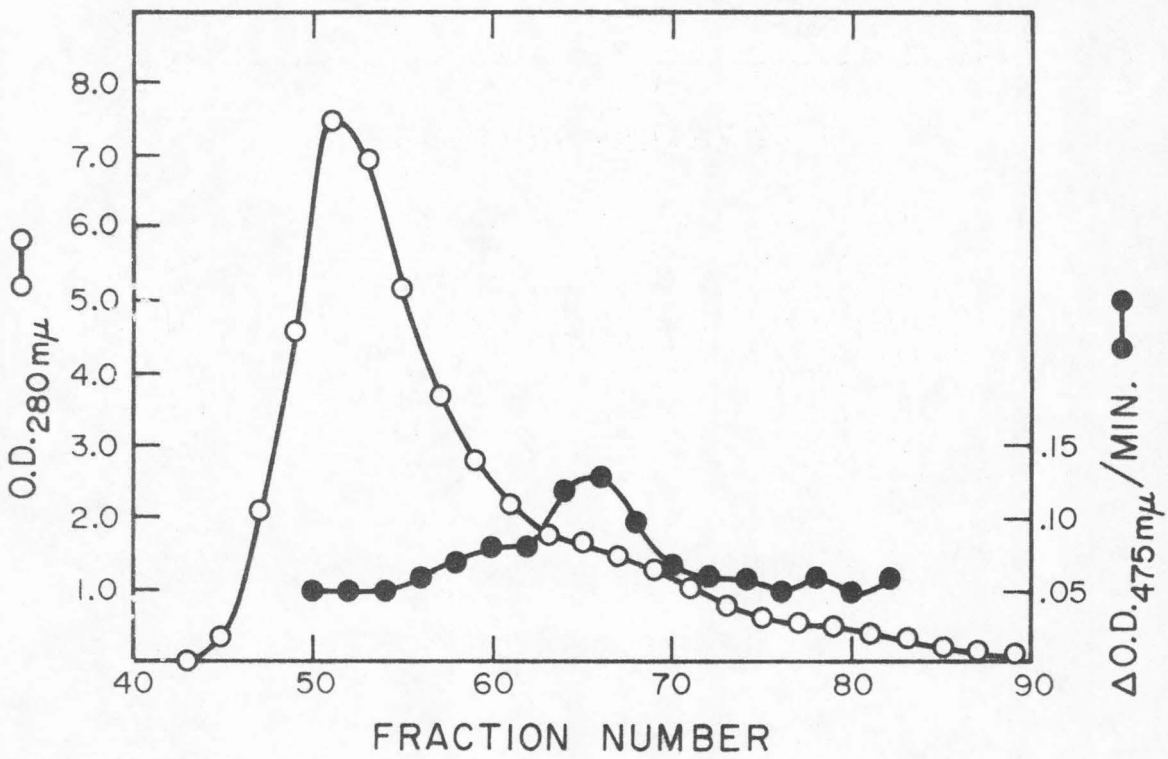

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
O.D.280mμ
.15
.10
.05
ΔO.D.475mμ/MIN.
40
50
60
70
80
90
FRACTION NUMBER

Figure 4. G-100 chromatography of crude A. The sample was crude A prepared from 10 g of 4 to 7 hour prepupae. The elution buffer was 0.01 M TrisHCl, pH 7.2. The elution rate was 0.43 ml/min. Fractions were collected at 20 minute intervals. 20 µl aliquots of each fraction were assayed in Assay II.

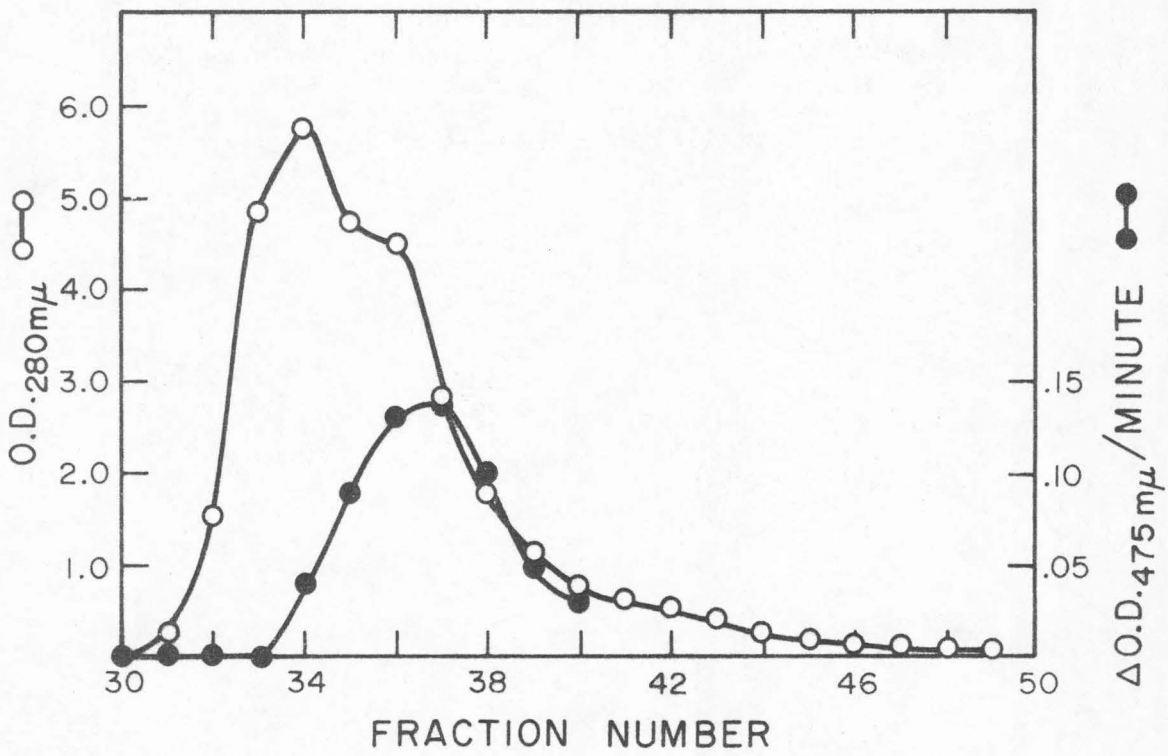
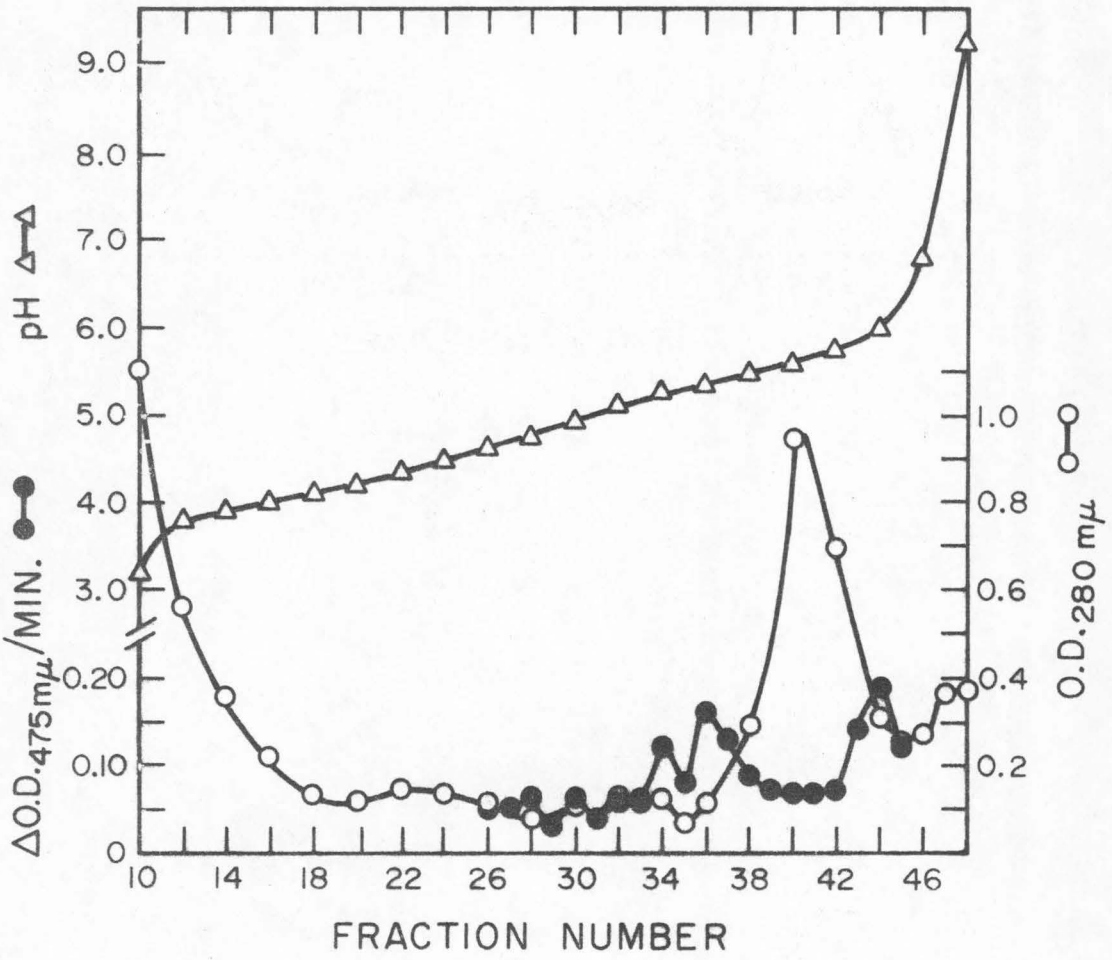

6.0
5.0
4.0
3.0
2.0
1.0
O.D.280mμ
.15
.10
.05
ΔO.D.475 mμ/MINUTE
30
34
38
42
46
50
FRACTION NUMBER

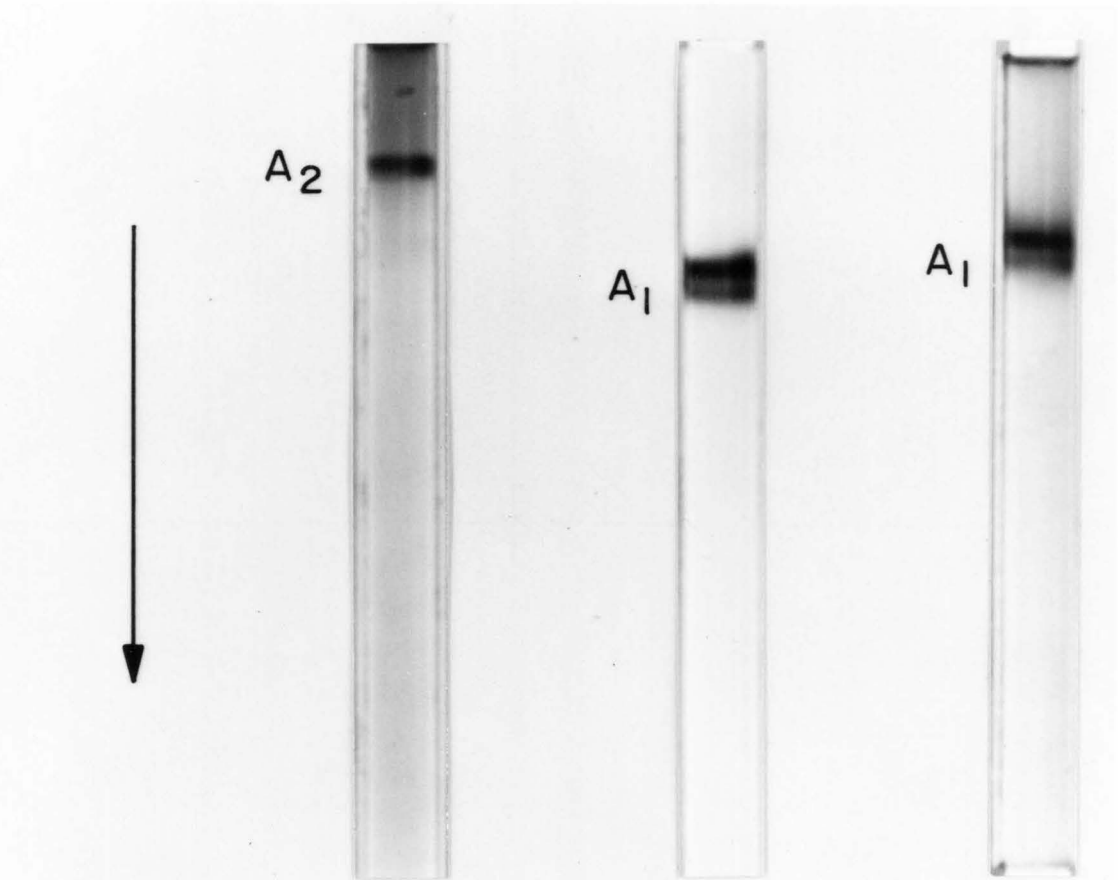
Figure 5. Preparative electrofocusing of the A components on a two pH unit gradient. The sample was prepared from 400 g of late third instar larvae (see Materials and Methods, section 5A), and had been previously purified by G-200 and G-100 chromatography. The conditions for electrofocusing were as described in Materials and Methods (section 9A). Focusing was carried out for 60 hours. 20 µl aliquots of each fraction were assayed in Assay I.


9.0
8.0
7.0
6.0
5.0
4.0
3.0
0.20
0.10
0
pH
ΔO.D.475mμ/MIN.
1.0
0.8
0.6
0.4
0.2
O.D.280 mμ
10
14
18
22
26
30
34
38
42
46
FRACTION NUMBER

the two fractions containing A component activity were assayed on polyacrylamide gels, the A_1 component was found in the pI 5.1 fraction, and the A_2 component was found in the pI 6.0 fraction (Figure 6). Note that the A_1 fraction contains two very closely spaced bands of A component activity. When samples prepared from prepupae by extraction in 37.5% saturated $(NH_4)_2SO_4$ (see Materials and Methods, section 5B.) were analyzed by electrofocusing, no A_2 component peak was found, indicating that A_2 is not extracted by this method.

A distinct advantage of the electrofocusing technique is the utilization of a relatively dilute sample which becomes concentrated as it is purified. This feature minimizes loss due to activation. The electrofocusing method does present one difficulty, long running time (48 to 72 hours). This problem has been solved and resolution improved by utilization of a preformed, expanded pH gradient in the following fashion (see Materials and Methods, section 9B.). First an equilibrium pH gradient is prepared by electrofocusing a high concentration of ampholytes without a protein sample. The gradient is collected in fractions and the tubes are stored which span a one pH unit range with the pI of A_1 in the center. In order to fractionate a protein sample, the first half of the preformed gradient is introduced into the column. The sample is then mixed with the tube which matches the isoelectric point of A_1 and intro-

Figure 6. Electrophoretic analysis of the A component fractions recovered from electrofocusing. The gels were developed for A component activity and incubated in the substrate indicated (see Materials and Methods, section 13). Gel number 1 shows the pI 6 fraction (fraction 44 in Figure 5). Gels 2 and 3 show the pI 5 fraction (fraction 36 in Figure 5). The sample applied to gel number 1 contained 115 µg of protein. The samples applied to gels 2 and 3 contained 25 µg of protein. The arrow at the left indicates the direction of migration.

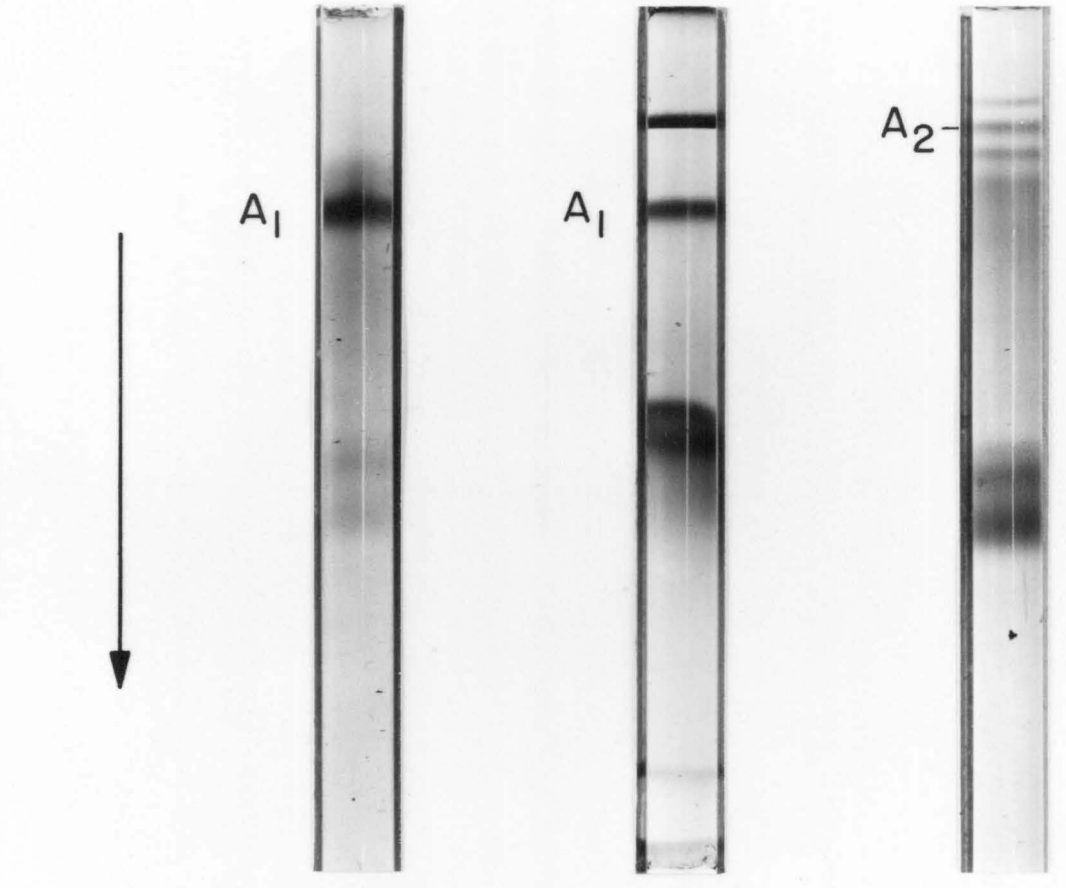

A2
A1
A1
Fraction: pI6 pI5 pI5
Substrate: DOPA DOPA TYR
Gel Number: 1 2 3

duced into the middle of the column. The remainder of the preformed gradient is layered on top of the sample. Sharp protein zones can then be focused in 12 to 15 hours with improved resolution. Some precipitation of contaminants occurs during focusing, but this does not interfere with the purification. Refrigeration of the column to 9°C. greatly reduces precipitation but does not affect the yield. The yield of the A_1 component is 40%. Analytical acrylamide gels of typical products from electrofocusing experiments in two pH unit gradients are shown in Figure 7. Note that the A_1 component is recovered with very few contaminants. The A_2 component, focusing somewhat closer to the main protein peak, is recovered in less pure form. The difference in the purity of the A_1 samples shown in Figure 7 (gels 1 and 2) is probably due to variation in the composition of the sample applied to the electrofocusing column and is not due to variability in the resolution of particular electrofocusing experiments. Re-electrofocusing a sample such as that illustrated by gel number 2 in **Figure** 7 does not result in improved purification. Because of the low risk of phenol oxidase activation and the excellent purification obtained, electrofocusing is an extremely useful technique for the A components.

D. Preparative Polyacrylamide Gel Electrophoresis.

Preparative electrophoresis on sieving polyacrylamide gels is a very useful method in the final stages of

Figure 7. Electrophoretic analysis of the A component fractions recovered from electrofocusing in two pH unit gradients (see Materials and Methods, sections 13 and 9A). The gels were stained for protein. Gels 1 and 3 show the pI 5 and pI 6 fractions recovered from the experiment shown in Figure 5. Gel 2 shows the pI 5 fraction obtained from a different experiment using 4 to 7 hour prepupae (see Materials and Methods, section 5B). The samples applied to gels 1 and 2 each contained 100 µg of protein. The sample applied to gel 3 contained 230 µg of protein. The arrow at the left indicates the direction of migration.


A1
A1
A2
Fraction: pI5 pI5 pI6
Gel Number: 1 2 3

purification of the A_1 component. A_1 can be eluted from such gels very reproducibly over a 30 minute period from 4.75 to 5.25 hours after beginning electrophoresis (see Materials and Methods, section 8. for conditions). The yield varies from 10% to 90%. If samples containing 250 units of A_1 activity (about 0.5 mg of protein) or less are used, excellent recovery can be expected. With samples of over 500 units (about 1.0 mg of protein or more), considerable activation and precipitation occurs at the spacer gel-separating gel interface, and yield is drastically reduced. Furthermore, if the gel is overloaded and activation occurs, poor purification is obtained. However, preparative electrophoresis is a valuable and reliable technique for small samples.

E. DEAE-cellulose Chromatography of A_2.

Although consistently poor recoveries prevented the use of DEAE-cellulose in the usual adsorption and elution fashion, conditions were found such that only contaminants were adsorbed to the column while the A_2 component passed through. This procedure has a yield of 90%; it is useful in the preparation of A_2.

F. Hydroxylapatite Chromatography of A_2.

Although it proved difficult to elute A_1 from hydroxylapatite, A_2 can be eluted readily in 0.3 M pyro-

phosphate with a yield of 80%. This step is useful at the final stages of A_2 purification.

6. Integrated Purification Schemes.

The procedures described above have been combined into two purification schemes for A_1 using larvae (Table 2) and 4 to 7 hour prepupae (Table 3). Both of these procedures have been carried out routinely. The A_2 component may be recovered in the first procedure only by passing the product obtained by electrofocusing over DEAE-Sephadex and hydroxylapatite as described above. The second method, as mentioned earlier, uses a higher specific activity starting material and involves considerably less risk of loss due to activation. The A_1 component is therefore somewhat easier to prepare. For this reason, as well as its abundance and its unambiguous definition by substrate specificity on analytical polyacrylamide gels, most purification work has been directed towards A_1. Analytical polyacrylamide gels of final products are shown in Figure 8. The products of both procedures (Tables 2 and 3) are referred to as "purified A_1" below.

7. Criteria of Purity and Properties of A_1.

A. SDS Polyacrylamide Gel Electrophoresis.

When analyzed on SDS polyacrylamide gels (144),

Table 2.

Purification of A_1 From 100 Grams of Larvae

Step	Total Soluble Protein (mg)	Total Units of A Component Activity	Specific Activity in Units per mg of Soluble Protein	Yield for Step (%)	Purification for Step
$(NH_4)_2SO_4$ precipitate	2500	13,700	5.5	-	-
G-200 chromatography	220	11,000	50	80%	9 X
G-100 chromatography	88	8,800	100	80%	2 X
Electro-focusing (2 pH unit gradient)	6.7	3,000	450	34%	4.5 X
Preparative Electro-phoresis	1.6	900	560	30%	1.25 X

Total yield relative to crude A = 6.5%

Total purification relative to crude A = 101 fold

Total purification relative to total soluble protein = 222 fold

Table 3.

Purification of A_1 From 10 Grams of Prepupae.

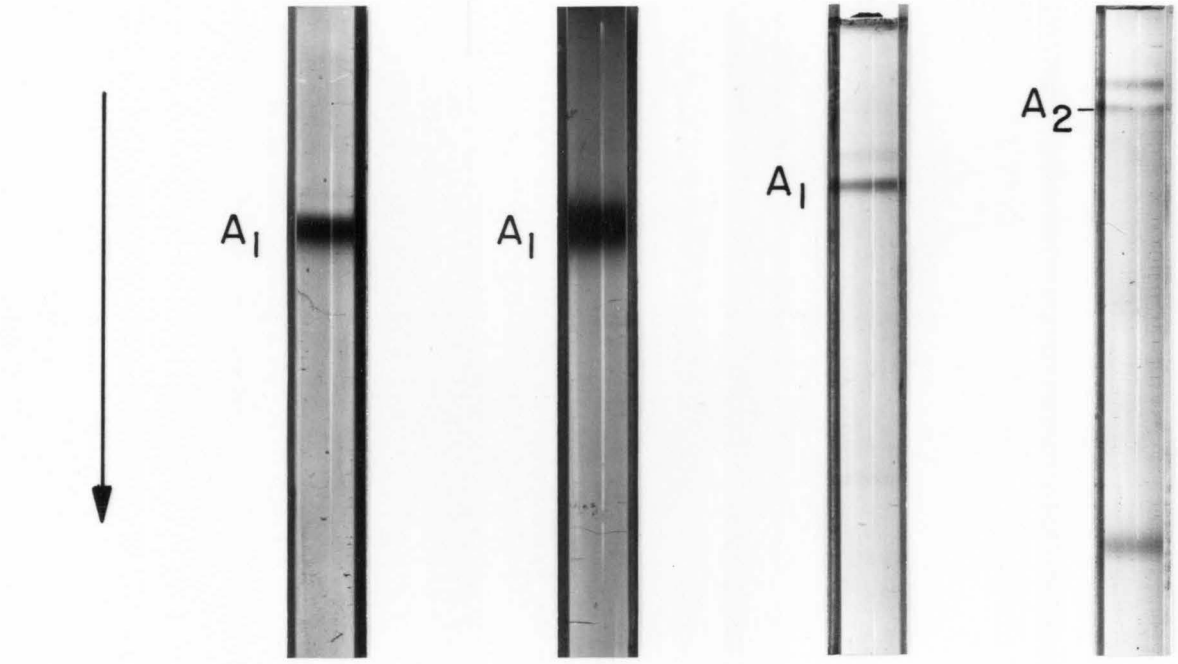
Step	Total Soluble Protein (mg)	Total Units of A Component Activity	Specific Activity in Units per mg of Soluble Protein	Yield for Step (%)	Purification for Step
$(NH_4)_2SO_4$ precipitate	230	2900	12.5	-	-
G-200 chromatography	23.2	2320	100	80%	8 X
Electro-focusing (expanded gradient)	2.1	1040	500	45%	5 X
Preparative Electro-phoresis	0.93	520	560	50%	1.1 X

Total yield relative to crude A = 18%

Total purification relative to crude A = 44 fold

Total purification relative to total soluble protein = 104 fold

Figure 8. Electrophoretic analysis of purified A components (see Materials and Methods, section 13). Gel 1 shows purified A_1 produced by the scheme shown in Table 2 (22 µg of protein); gel 1 was stained for protein. Gel 2 shows the same sample as gel 1 (5 µg of protein), but the gel was developed for A component activity. Gel 3 shows the product of the purification scheme for A_1 outlined in Table 3 (10 µg of protein); gel 3 was stained for protein. Gel 4 shows purified A_2 prepared as described in Results (section 6) (10 µg of protein); gel 4 was stained for protein. The arrow at the left indicates the direction of migration.


A1
A1
A1
A2
Gel Number: 1
2
3
4

purified A_1 gives two very closely spaced bands of unequal intensity, the more rapidly moving band being the less intense (Figure 9). By reference to a calibration curve based on standard proteins of known molecular weight (see Figure 10), the molecular weight of the major band is 77,000 daltons. No sign of dissociation of the form seen on SDS gels was ever observed. The various conditions of sample preparation (see Materials and Methods, section 15.) such as heating at 86°C. in 8 M urea did not affect the mobility of A_1 on SDS gels.

B. Band Sedimentation.

The sedimentation of purified A_1 was observed in the analytical ultracentrifuge (Figure 11). An $s_{20,w}$ of 8.2s was obtained (assuming a partial specific volume of 0.70 ml/g). No minor components were observed. A contaminant present in 25% of the concentration of A_1 could have been detected.

C. Gel Electrofocusing.

Samples of purified A_1 were analyzed by electrofocusing in polyacrylamide gels (Figure 12) (145). Two closely spaced protein bands were observed. The more acidic band stained darkest, and the ratio of staining intensities of the two bands appeared to be the same as the two bands observed on SDS gels (see section 7A. above). Both bands

Figure 9. Electrophoretic analysis of purified A_1 on an SDS polyacrylamide gel (144) (see Materials and Methods, section 15). The sample was 10 µg of purified A_1 prepared according to Table 2. The sample was applied in 0.01 M phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% β-mercaptoethanol. Before application to the gel the sample was incubated at 37° for 2 hours. The pattern shown in the photograph is unaffected by any of the more violent denaturing conditions tested. The arrow at the left indicates the direction of migration.

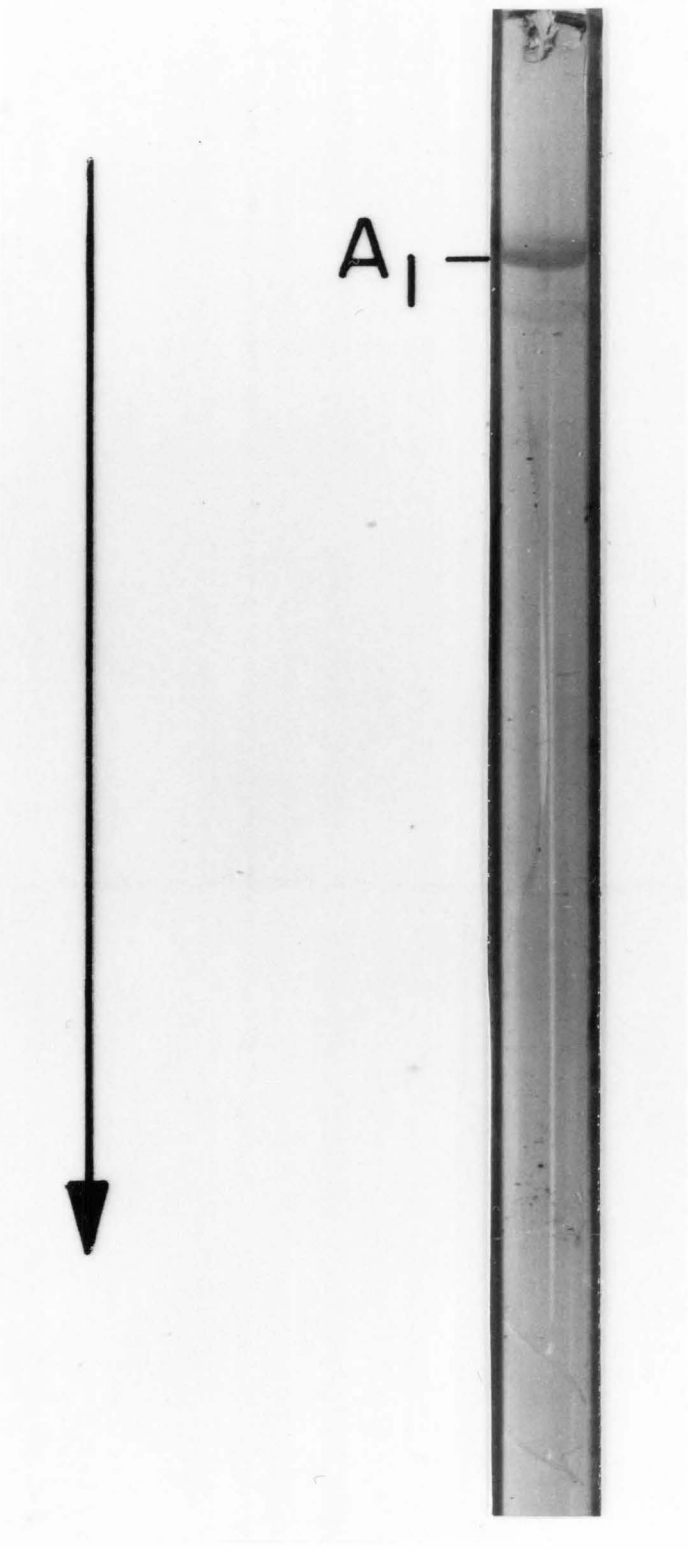

A1

Figure 10. The mobility of standard proteins and A_1 on SDS polyacrylamide gels (144) (see Materials and Methods, section 15). The mobilities of 10 µg samples of several standard proteins were determined relative to bromphenol blue. The samples were applied to the gels in 0.01 M phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% β-mercaptoethanol after a 2 hour incubation at 37°C. The mobility of A_1 (see Figure 9) is plotted for comparison.

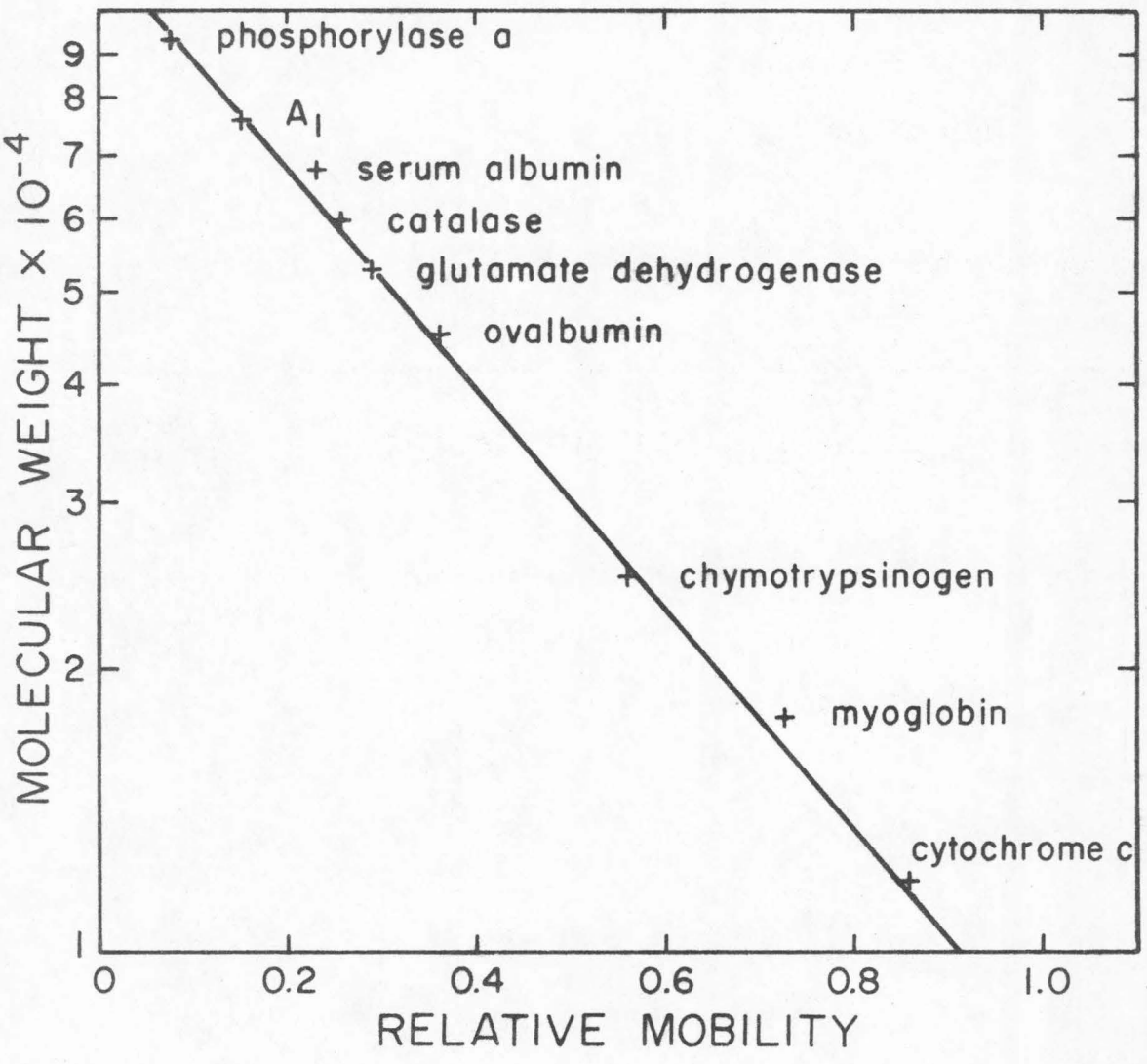
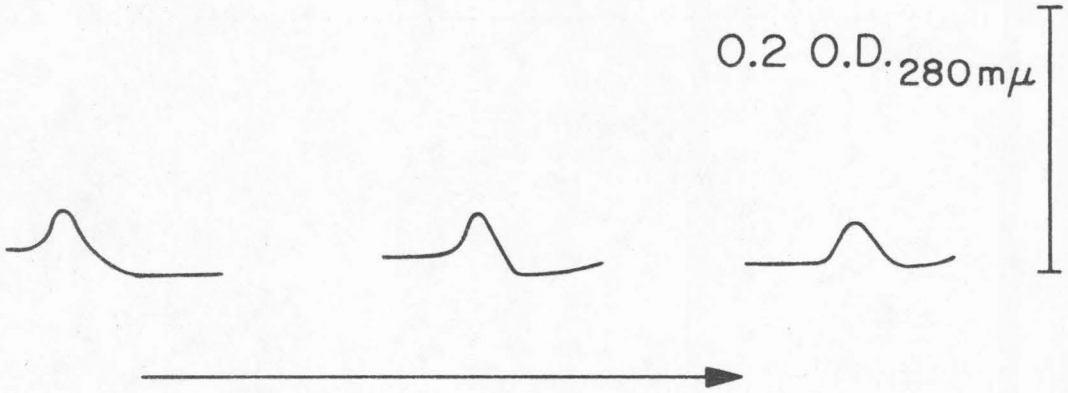

phosphorylase a
A1
serum albumin
catalase
glutamate dehydrogenase
ovalbumin
chymotrypsinogen
myoglobin
cytochrome c
MOLECULAR WEIGHT x 10-4
9
8
7
6
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 11. The sedimentation of purified A_1 in the analytical ultracentrifuge. The sample was 15 µg of A_1 prepared according to the scheme presented in Table 3. The centrifugal conditions were as described in Materials and Methods (section 17). Rotor speed was 52,000 r.p.m. The recordings shown at the top were taken at 16 minute intervals. The arrow below the tracing shows the direction of sedimentation and the bar at the right shows the 0.2 $O.D._{280\ m\mu}$ calibration. The lower portion of the figure shows the plot of the log of the distance of the peak from the center of rotation versus time.


0.2 O.D.280mμ

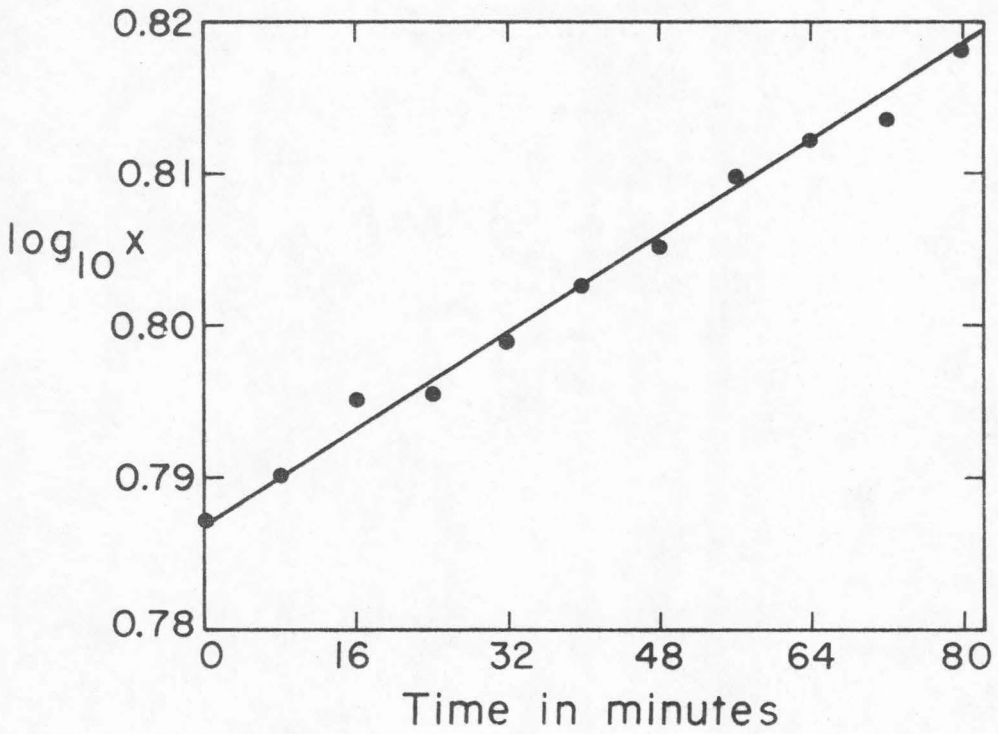
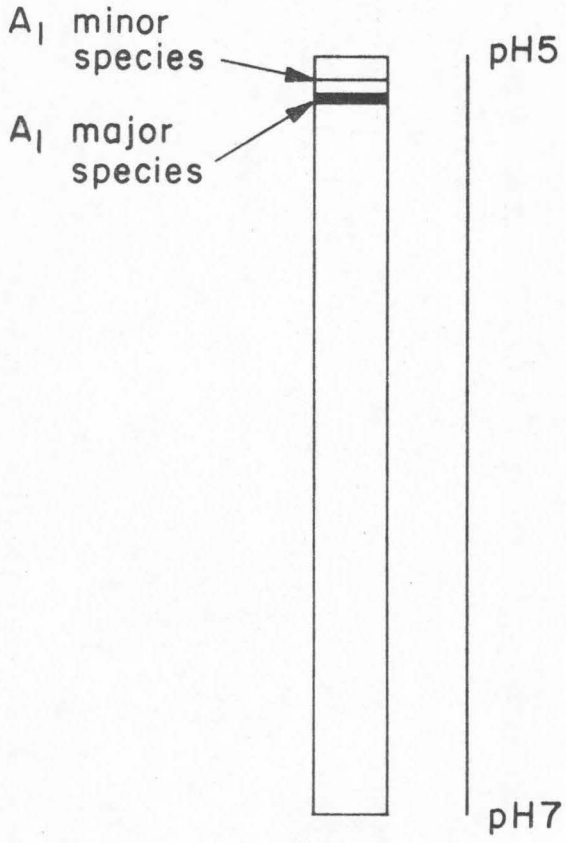

0.82
0.81
0.80
0.79
0.78
log10 x
0
16
32
48
64
80
Time in minutes

Figure 12. Analysis of purified A_1 by analytical electrofocusing (145) (see Materials and Methods, section 16). The figure shows a drawing of an analytical electrofocusing gel (the original could not be photographed clearly). The sample was 14 µg of purified A_1 prepared according to Table 2. The gel was stained for protein. The bar at the right indicates the direction of the pH gradient.


A_1 minor species
A_1 major species
pH5
pH7

had A component activity.

D. Copper Content.

The total copper content of A_1 was analyzed by the oxalyl dihydrazide method (148) which can detect as little as 0.1 µg of copper (see Materials and Methods, section 21). The standard curve for this determination is shown in Figure 13. Duplicate samples of purified A_1 (94 µg of protein per sample, prepared according to the scheme outlined in Table 3) contained 0.15% copper (corresponding to 0.11 $O.D._{540\ m\mu}$). Duplicate samples of ebony heavy band phenol oxidase (510 µg dry weight per sample; prepared by A. Blumenthal) contained 0.14% copper (corresponding to 0.46 $O.D._{540\ m\mu}$).

E. Lipid Content.

Since active phenol oxidase was reported to contain lipid (126), duplicate samples of purified A_1 (25 µg of protein per sample, prepared according to Table 3) were subjected to alkaline hydrolysis, acidified, and methanol extracted. The methanol extract was assayed for fatty acids by a very sensitive method (146) which can detect as little as 5 nanomoles of fatty acid (see Materials and Methods, section 18). The standard curve for this assay is shown in Figure 14. Fatty acids could not be detected in purified A_1. The limits of sensitivity of the assay and the significance

Figure 13. The standard curve for the total copper determination (148) (see Materials and Methods, section 21). The abscissa shows the copper concentration of the assay mixture in µg/ml. The copper standards were prepared from clean copper wire. Duplicate 94 µg samples of purified A_1 (prepared according to Table 3) gave $O.D._{542\ m\mu}$ values of 0.11 O.D. Duplicate 510 µg samples of ebony heavy band phenol oxidase (prepared by A. Blumenthal, 126) gave $O.D._{542\ m\mu}$ values of 0.46 O.D.

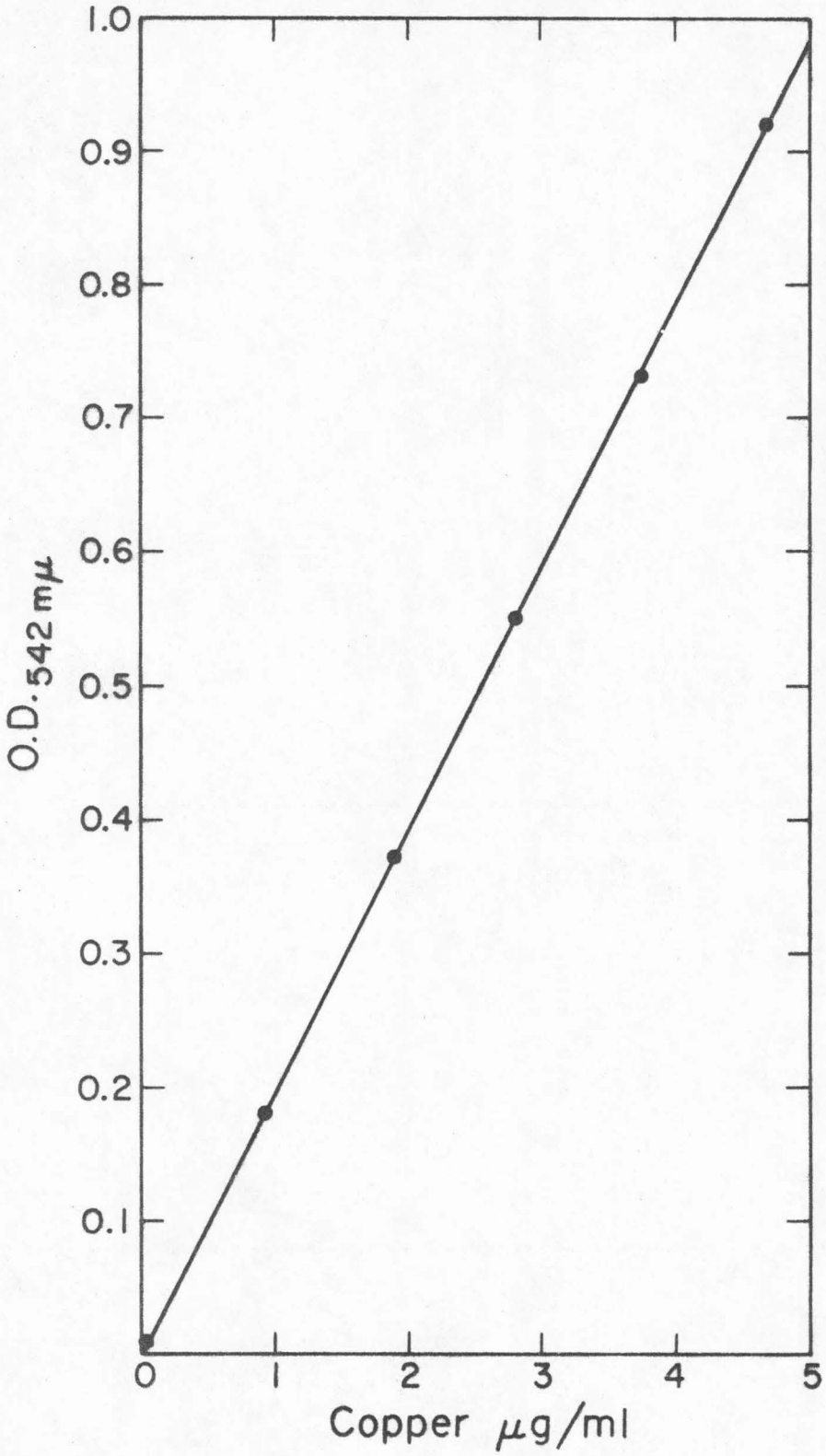
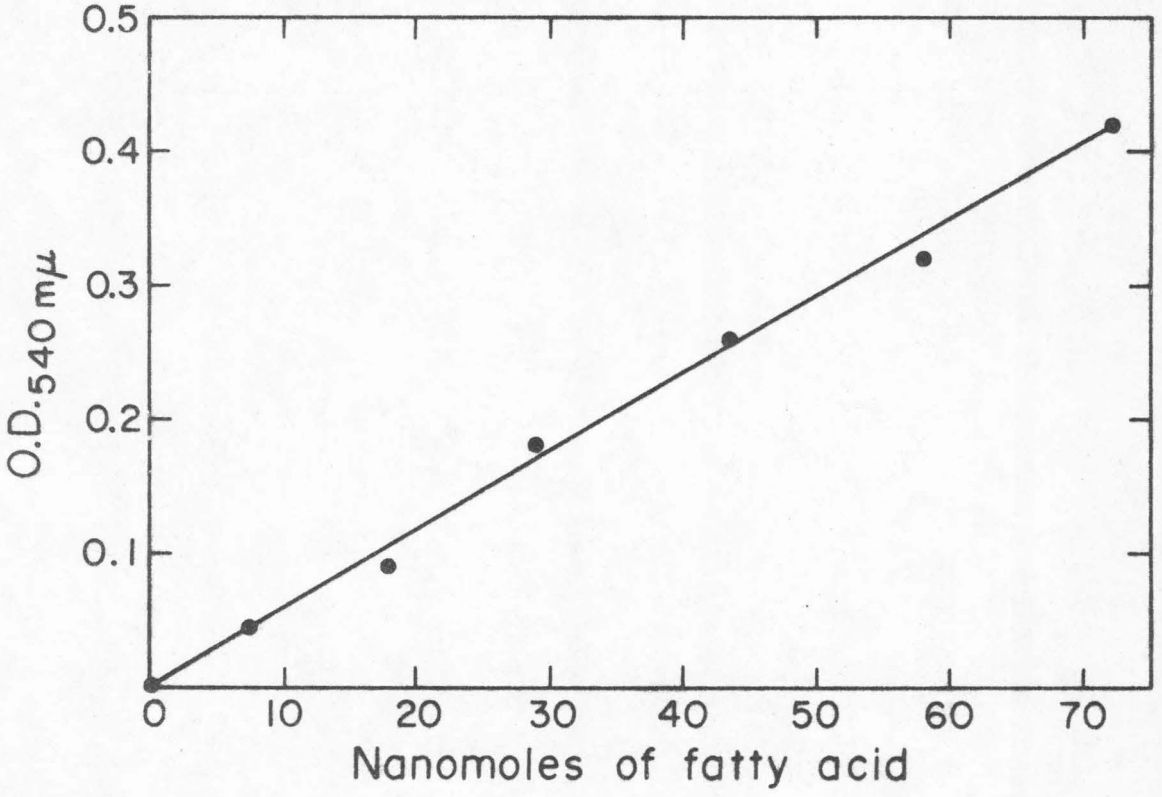

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
O.D. 542 mμ
0
1
2
3
4
5
Copper μg/ml

Figure 14. The standard curve for the fatty acid determination (126) (see Materials and Methods, section 18). The abscissa shows the total quantity of fatty acid in the assayed sample. The standards were prepared by serial dilution of a lauric acid solution in chloroform. Duplicate samples of A_1 (25 µg of protein prepared according to Table 3) gave baseline O.D.$_{540\ m\mu}$ readings.


0.5
0.4
0.3
0.2
0.1
O.D.540 mμ
0
10
20
30
40
50
60
70
Nanomoles of fatty acid

of this result are discussed below.

F. Phosphate Content.

Since active phenol oxidase was reported to contain phosphate (126), duplicate samples of purified A_1 (25 µg of protein per sample, prepared according to Table 3) were subjected to perchloric acid digestion and analyzed for phosphate (147) (see Materials and Methods, section 19). Non-digested samples were used to control for the presence of inorganic phosphate. The standard curve for this analysis is shown in Figure 15. One nanomole of phosphate could be detected. No organic or inorganic phosphate could be detected in purified A_1. The limits of sensitivity of this determination and its significance are discussed below.

G. Amino Acid Analysis.

The partial amino acid analysis of purified A_1 is given in Table 4. An unknown peak with a retention time similar to that of lysine was not identified. The amino acid analysis of ebony heavy band phenol oxidase is shown for comparison (126).

8. Enzymatic Properties of Purified A_1.

A. Lack of Phenol Oxidase Activity.

Purified A_1 has no phenol oxidase activity which can be measured by the usual spectrophotometric assay. Phenol oxidase activity cannot be detected in analytical polyacryl-

Figure 15. The standard curve for the phosphate determination (147) (see Materials and Methods, section 19). The abscissa shows the total quantity of phosphate in the assay mixture in nanomoles. The standards were prepared by serial dilution of a solution of inorganic phosphate. Duplicate samples of A_1 (25 µg of protein prepared according to Table 3) gave baseline readings.

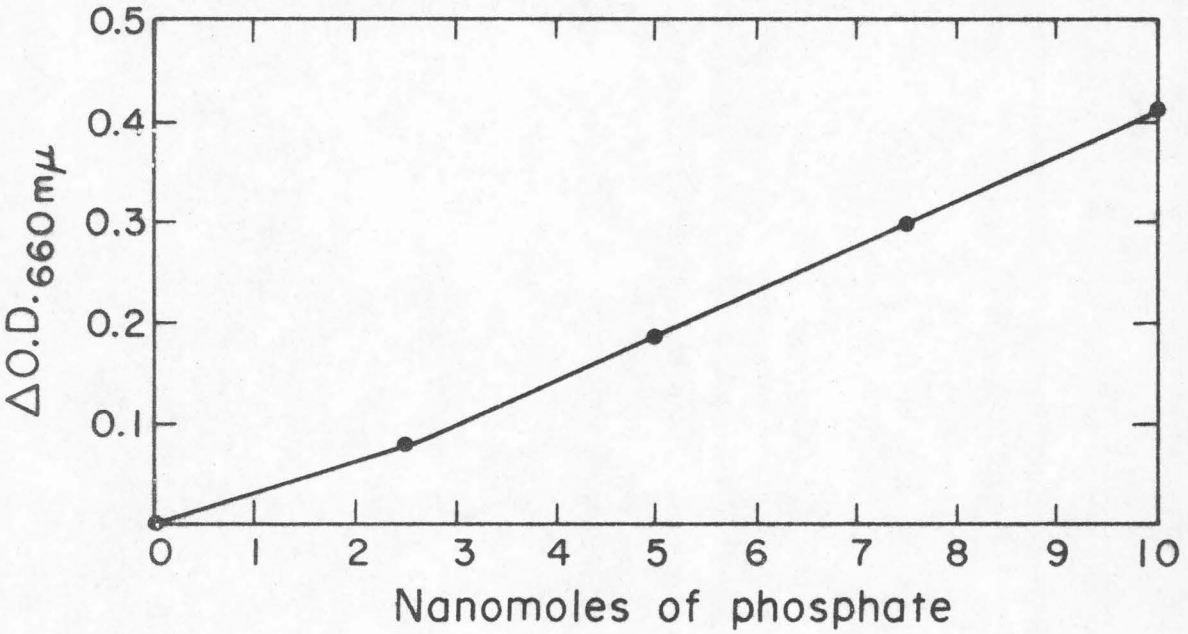

0.5
0.4
0.3
0.2
0.1
ΔO.D. 660 mμ
0
1
2
3
4
5
6
7
8
9
10
Nanomoles of phosphate

Table 4.

Partial Amino Acid Analysis of Purified A_1 and Banded Phenol Oxidase.
(moles %)

Amino Acid	A_1*	Phenol Oxidase**
ASP	12.6	9.6
THR	5.7	7.4
SER	5.6	5.4
GLU	9.8	11.9
PRO	4.2	5.9
GLY	12.7	12.2
ALA	10.8	7.5
VAL	6.5	3.6
MET	0.7	1.4
ILE	4.5	4.7
LEU	8.2	7.2
TYR	1.4	2.8
PHE	4.1	4.5
HIS	1.8	1.7
LYS	3.7	7.6
ARG	5.7	4.8
UNKNOWN	-	

*Purified A_1 was prepared according to Table 2.

**The data for banded phenol oxidase are taken from Blumenthal, Table 8 (126).

The analyses were carried out according to Materials and Methods (section 22).

amide gels of purified A_1 without incubation in crude S.

B. Activation by α-chymotrypsin.

When solutions of purified A_1 (25 µg/ml) were incubated for 15 minutes at 20°C. with α-chymotrypsin at concentrations up to 1 mg per ml in 0.01 M TrisHCl, pH 7.2, no phenol oxidase activity was observed in the usual assay. However, after prolonged incubation (60 minutes) in DOPA, some dopachrome formation was observed. This level was far below the useful range of the phenol oxidase assay and must represent a very small fraction of the potential activity.

C. Lack of Activation With Purified S.

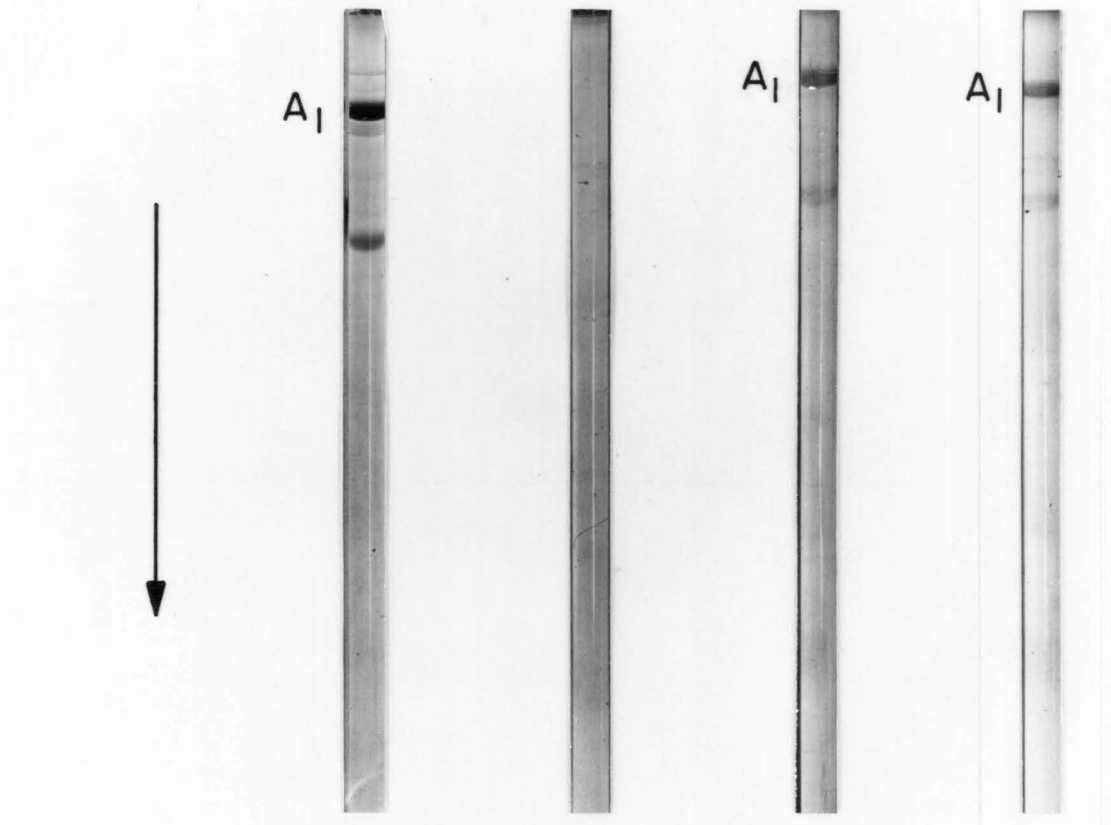
Purified A_1 has failed to produce phenol oxidase activity on incubation with purified S* in many experiments over a wide concentration range. Purified S will not activate purified A_1 in the electrophoretic assay. The possible effect of purified S on the molecular weight of A_1 was tested in the following fashion. Purified S was incubated with partially purified A_1 (material which had been carried through all but the final step of the scheme given in Table 3). Aliquots were removed from the incubation mixture after 30 and 60 minutes of incubation. Upon removal from the mixture the "reaction" was stopped by the addition of SDS, and

---

*Purified S was prepared by Mr. D. Seybold.

the samples were analyzed by SDS gel electrophoresis. No alteration in the molecular weight of A_1 could be detected (Figure 16).

Figure 16. Demonstration of the lack of effect of purified S on the molecular weight of A_1 as determined by SDS gel electrophoresis (144) (see Materials and Methods, section 15). The purified S was prepared by Mr. D. Seybold. The partially purified A_1 was material which had been carried through all but the final step of the scheme shown in Table 3. All gels were stained for protein. Gel number 1 shows 12 µg of partially purified A_1 alone. Gel number 2 shows 5 µg of purified S alone. Note that no dark bands are visible in gel number 2 which might obscure bands in the mixture applied to the following gels. Gel number 3 shows a mixture of 6 µg of partially purified A_1 and 5 µg of purified S preincubated for 30 minutes at 4°C. in 0.01 M TrisHCl, pH 7.2 before the addition of SDS. Gel 4 is the same as gel 3, except that a 60 minute preincubation was used. The A_1 band has the same mobility on every gel. All samples were applied to the gels in 0.01 M phosphate buffer, pH 7.0 containing 0.1% SDS and 5% β-mercaptoethanol after incubating at 50°C. for 1 hour. The arrow at the left indicates the direction of migration.



Sample:	A_1	S	A_1 + S	A_1 + S
Preincubation:	—	—	30 min	60 min
Gel Number:	1	2	3	4

DISCUSSION

It has been possible to prepare the A components of the latent Drosophila phenol oxidase in stable, purified form. Although they are normally converted to active phenol oxidase in crude extracts, reliable methods for purifying the A components have been developed. The A_1 component was shown to be free of major contaminants by analytical polyacrylamide gel electrophoresis, analytical electrofocusing, and band sedimentation.

The presence of three A components was described by Mitchell and Weber (120). The A_1 component gives rise to an active phenol oxidase which can act on both monophenols and diphenols. The A_2 and A_3 components give rise to oxidases which can act only on diphenols. The A components can be distinguished both by their electrophoretic mobilities and by their solubilities in $(NH_4)_2SO_4$. The present work has verified the presence of three A components in crude extracts. However, only the A_1 and A_2 components were obtained upon fractionation of crude extracts. Several explanations may be proposed to resolve this conflict. The A_3 component may be an electrophoretic artifact found only in crude fractions. An alternative explanation is that the A_3 component is either labile or not extracted under the conditions used. The latter possibility is quite likely in the case of preparations

from prepupae where A_2 was not extracted. A_3 should be even less soluble than A_2 under those conditions. The electrophoretic assay for the A components does not provide quantitative data; it is therefore possible that the A_3 component is simply not present in quantities which are detectable by the A component assay. Further analysis of the 37.5 to 41% saturated $(NH_4)_2SO_4$ fraction might clarify this question.

The present work has shown that A_1 and A_2 are in fact chemically distinct proteins. Their isoelectric points are nearly a full pH unit apart, and the separated components show no sign of interconversion. This finding supports the model, further discussed below, that the proportions of the A components control the substrate specificity of the active phenol oxidase. It also represents an addition to the list of diphenol oxidases without monophenol oxidase activity strengthening the concept that the enzyme plays an essential role in the oxidation of monophenols.

The A_1 component is a protein with a molecular weight of approximately 77,000 daltons as determined by SDS gel electrophoresis and gel filtration. The copper content suggests the presence of two copper atoms per molecule as in the phenol oxidases of other organisms. This result lends considerable credence to the hypothesis that A_1 contributes active sites to the phenol oxidase molecule. Although there was no indication that A_1 dissociates in SDS, the possibility that A_1 is composed of subunits cannot be discarded at the

present time. The most violent conditions tested (boiling in 1% SDS) are sufficient to dissociate most proteins. However, the possibility remains that A_1 is an unusually stable multimer. It should be recalled that mushroom phenol oxidase is unusually stable to dissociation in SDS (9, 18). It is perhaps of greater relevance that the Bombyx phenol oxidase proenzyme, which is thought to be a multimer, does not dissociate in SDS (115).

An interesting question is raised by the presence of microheterogeneity in purified A_1 preparations. Microheterogeneity was first indicated by analytical electrofocusing and analytical electrophoresis. Preparative electrophoresis confirmed the presence of a minor species which appears in the same proportion in all analytical techniques. The major species is very slightly larger and more acidic than the minor species. Both forms have A component activity. The data do not permit an accurate estimate of the quantity of the minor species which is present, but from its staining intensity on electrofocusing gels, the minor component probably represents less than one quarter of the total purified A_1. Two classes of explanations may be proposed to explain the presence of the minor form. The first is that neither form is chemically derived from the other. They might represent true heterogeneity or, perhaps, genetic variation in the Oregon R stock. Analytical electrofocusing of extracts prepared from single larvae could

be used to refute a genetic explanation of microheterogeneity. Another class of explanations which can be considered is that the multiplicity of A_1 forms is the result of partial activation. This possibility which seems rather likely will be discussed below.

It appears that the S, P, and A_1 components constitute a minimal activating system (123). Purified S has no activating effect on purified A_1, and activation evidently requires the action of both S and P. The possibilities are presented schematically in Figure 17. If S and P act sequentially, and the activation of A_1 proceeds through more than one step, then the minor component in purified A_1 could be the product (A_1^*) of one step in the activation reaction. On this view, it would be required that S act on A_1 before P since all preparations would have been exposed to P to some extent. Yet purified A_1 is not activated by purified S. Of course, the putative intermediate A_1^* might be very unstable. An objection to the first possibility presented in the diagram is the absence of any effect of purified S on the molecular weight of A_1. However, the hypothetical action of S on A_1 might not involve a large alteration in the molecular weight of A_1.

The possibility remains that S and P interact to produce the actual activator molecule which activates A_1 in a single step. This is the second model presented in Figure 17. According to this model, the actual activating factor is an

Figure 17. Two models for the activation of A_1. A_1^* represents the hypothetical product of the action of S on A_1. A_1^{**} represents the hypothetical immediate precursor to the phenol oxidase aggregate.

Multistep Activation of A_1

Step 1: S activation $\quad Pre\ S \xrightarrow[S]{S\text{-activator}} S$

Steps 2 & 3: A_1 activation $\quad A_1 \xrightarrow{S} A_1^* \xrightarrow{P} A_1^{**}$

Step 4: Phenol oxidase aggregation

$nA_1^{**} \longrightarrow$ Phenol oxidase

Single Step Activation of A_1

Step 1: S activation $\quad Pre\ S \xrightarrow[S]{S\text{-activator}} S$

Step 2: Aggregation of S and P $\quad S + P \longrightarrow S - P$

Step 3: Activation of A_1 $\quad A_1 \xrightarrow{SP} A_1^{**}$

Step 4: Phenol oxidase aggregation

$n\,A_1^{**} \longrightarrow$ Phenol oxidase

aggregate of S and P. A variation of this model may be considered. The interaction of S and P may be the interaction of enzyme and substrate, the product of the reaction being the actual activating factor. Recent results show that the activation of crude A_1 by purified S and purified P is accelerated by mixing and preincubating S and P before the addition of A_1 (123). This result strongly indicates that S and P interact in some fashion. Further analysis of the kinetics of the interaction of S and P should permit discrimination between the alternatives of aggregation and enzymatic activity. Unfortunately at the present time, the purified S and P components are not available in sufficient quantity to permit the study of their interaction by the usual analytical techniques. If the actual activating factor is the product of the interaction of S and P, and the actviation of A_1 proceeds in a single step, then the minor form of A_1 might correspond to the hypothetical precursor to aggregated phenol oxidase, A_1^{**}. A_1^{**} might not be fully active until aggregated. The lower molecular weight of the minor form, indicated by SDS gel electrophoresis may be interpreted as support for the hypothetical proteolytic mechanism of activation discussed below.

The schemes proposed in Figure 17 do not specify mechanisms for any of the steps involved in the activation reaction. There are at least three distinct processes to consider, the activation of Pre-S, the activation of A_1, and

the aggregation of phenol oxidase. The information available at present does not allow any definitive statements about these reactions. However, the experimental data do limit the possible mechanisms, and certain alternatives can be considered. Many authors have advanced the hypothesis that the activation of insect phenol oxidase involves limited proteolysis (104, 107, 116, 127). This mechanism, which accounts for several well known enzyme activation phenomena, may explain both the activation of Pre-S and the activation of A_1 (150). Although Blumenthal found that in Drosophila, phenol oxidase activation is essentially insensitive to diisopropyl fluorophosphate, this result serves merely to rule out serine proteases (120). The phenol oxidase precursors of Calliphora, Bombyx, and Drosophila can be partially activated by treatment with α-chymotrypsin (107, 116). Certainly, this information does not directly support the hypothesis of limited proteolysis. It is quite easy to imagine that limited proteolysis might expose a sterically blocked active site or bring about a conformational change which would result in activation. Such alterations in the proenzyme molecule might be the result of mechanisms other than proteolysis. In Bombyx, the product of artificial proteolytic activation was analyzed (116). It had a molecular weight which was about half that of the proenzyme, and it did not undergo the normal aggregation reaction. In spite of these criticisms, the limited proteolytic mechanism is still the most attractive

hypothesis which has been proposed to date. As soon as purified S and purified P are available in sufficient quantity, it should be possible to search for an intermediate (A_1^{**} in Figure 17) derived from A_1 by one or more proteolytic cuts in the polypeptide chain of the A_1 molecule.

Analysis of the enzymatically active product of the activation of purified A_1 by purified S and purified P should further clarify the relationship of A_1 to the aggregates formed in crude extracts studied by Mitchell *et al.* and Blumenthal (125, 126). The first question to consider should be easy to solve: does purified A_1 aggregate when activated by purified S and purified P? One certainly would expect aggregation to occur in the purified activating system, but the aggregation reaction may require the catalytic action of an additional factor or the presence of A_2 or A_3. The aggregation of fibrin following the limited proteolysis of fibrinogen may be somewhat analogous to the aggregation of phenol oxidase (150). If aggregation does occur in the purified system, then the molecular weight and dissociability of the aggregate could be determined. Since the presence of low molecular weight crosslinking agents could be rigorously excluded, the purified system could be used to test the possibility proposed by Mitchell and further discussed by Blumenthal that the active phenol oxidase is stabilized by quinone crosslinks. The effect of the addition of controlled amounts of specific phenolic substances on the aggregation

of phenol oxidase could be determined. The alternative possibility, that covalent crosslinking occurs, as in fibrin, without the presence of a low molecular weight crosslinking agent, could be tested in the purified system. Eventually it should be possible to prepare the A_2 and A_3 components in purified form. From the properties of the "complete" purified system, it would be possible to test the effect of the proportions of the A components on the substrate specificity, aggregation state, and density of the active enzyme.

Although the properties of the phenol oxidase produced in the purified system have not yet been determined, it is possible to compare the properties of purified A_1 with those of banded phenol oxidase as reported by Blumenthal (126). In the following discussion it should be recalled that Blumenthal studied the phenol oxidase prepared from the ebony-1 strain while the present work has utilized the Oregon R stock. The amino acid composition of purified A_1 differs from that of banded phenol oxidase. The differences are probably due to the presence of other A components in the banded enzyme. The possibility that Blumenthal's preparations were contaminated by non-enzymatic proteins cannot be definitively ruled out, but he did find that ^{14}C labelled E. coli proteins added to the activation mixture did not contaminate the banded enzyme. The copper content of purified A_1 (0.15% on the basis of protein content determined by the Lowry method) is nearly the same as that of banded phenol

oxidase (0.14% on the basis of dry weight). If the figure for banded phenol oxidase is computed with respect to Blumenthal's estimate of the weight per cent of amino acids in banded phenol oxidase (75%), then the copper content of the banded enzyme is 20% higher than that of A_1. Of course, the copper content of banded phenol oxidase could be influenced by the presence of protein components other than A_1. In any case, the two figures are in the same range and are comparable to the figures reported for other phenol oxidases (compare, for example 0.15% copper for A_1 to 0.15 - 0.16% for the Bombyx proenzyme (115)).

Blumenthal estimated that his preparations of banded phenol oxidase contained 25 to 35% lipid of which about one quarter was phospholipid, the rest being non-polar lipid (126). Although he did demonstrate the presence of fatty acids in his preparations, his quantitative figures are based on the difference between the total weight of his samples and the fraction of the total weight accounted for by amino acids. He did not make a direct, quantitative determination of the fatty acid composition. Further evidence suggesting the involvement of lipoidal material in phenol oxidase structure was provided by Lewis and Lewis (127, 128). They found that delipidation of crude extracts lengthened the lag period preceding activation. Replacement of the removed lipid with endogenous lipid or certain exogenous lipids (cod liver oil, peanut oil, and cottonseed oil) reduced the lag period to a normal level. They also

showed that chloroform treatment of crude extracts markedly increased activation rates. Because of the interesting possibilities raised by the above experiments, and because lipid is a well known constituent of insect cuticle, samples of purified A_1 were analyzed directly for fatty acid content following alkaline hydrolysis. No fatty acids were detected. It can be seen from Figure 13 that quantities as small as 10 nanomoles of fatty acid could have been easily detected although as little as one half of this figure probably would have been seen. This puts the lower level of sensitivity of this determination at 8 to 16% fatty acids by weight (assuming a fatty acid with a molecular weight of 200 daltons). This assay would therefore have detected fatty acids down to about one third the level reported for banded phenol oxidase. Blumenthal reported that banded phenol oxidase contained phosphate at a level of 8.4×10^{-8} moles/mg. The assay shown in Figure 14 could detect as little as 10^{-9} moles of phosphate or 4×10^{-8} moles/mg. No phosphate was detected at this level which is about one half the amount reported by Blumenthal. It would appear then that A_1 does not contain large quantities of neutral lipid or phospholipid. However, the presence of these substances in small amounts cannot be ruled out. What, then accounts for the presence of lipid in banded phenol oxidase? Two major possibilities may be considered. In the first case, lipid may be contributed by a protein component other than A_1. At the present time

there is no evidence which suggests the existence of such a lipoprotein precursor to phenol oxidase. Another explanation which may be considered is that since active phenol oxidase occurs as a high molecular weight aggregate, lipid could be adsorbed to the aggregate or even physically trapped within it during isolation. This could resemble an adsorption reaction which might occur in cuticle. Perhaps such a process explains the enhancement of phenol oxidase activation by added lipid described by Lewis and Lewis (127, 128). This effect could be reinvestigated in the purified system where careful control of the composition of the activation mixture would be possible. Any tendency of the active enzyme to adsorb lipid should be easy to demonstrate.

It is possible at this point to stress the similarity of the various insect phenol oxidases which have been studied. This similarity is particularly well illustrated by the parallel between the Bombyx proenzyme and A_1 (115). Both are proteins of similar molecular weight and isoelectric point. They have nearly the same electrophoretic mobility on analytical polyacrylamide gels and SDS gels. The copper content is nearly the same in both cases. The Bombyx active enzyme aggregates as in Drosophila. The only notable difference reported is the greater sensitivity of the Bombyx proenzyme to activation by α-chymotrypsin. The Bombyx activating system has not been analyzed in detail, but those features which have been reported resemble the Drosophila

enzyme closely. It will be recalled that the Drosophila S component crossreacts with the Calliphora proenzyme (124). It seems safe to predict that as further details of the Bombyx and Drosophila systems are elucidated, the pattern of similarity will be extended.

The availability of purified A_1 should permit further clarification of several problematical aspects of Drosophila phenol oxidase in addition to those discussed above. In particular, the question of function is most important. Assuming the purity of banded phenol oxidase, late third instar larvae contain approximately 1.5 mg of phenol oxidase per gram of animals (calculated from figures in reference 120). This represents 2.7% of the total soluble protein of the organism. Of course it is just this abundance which permits the purification of A_1 from what is really a small amount of tissue (20 g of prepupae) by the usual standards of protein purification. The abundance of phenol oxidase is consistent with its proposed role as a major structural protein. Utilization of purified A_1 should permit a definitive test of this hypothesis. Radioactive A_1 could be prepared by labelling purified A_1 *in vitro* with a suitable agent, perhaps tritium or ^{131}I. Labelled A_1 could then be injected into larvae at various stages of development. The fate of the injected radioactivity could be followed by either autoradiography or liquid scintillation counting. If A_1 is incorporated into cuticular structures, an obvious

incorporation of radioactivity into cuticle should be observed. This method could also be used to define precisely the time of incorporation of phenol oxidase into cuticular structures. Of course, this approach depends upon the validity of the assumption that radioactive A_1 injected into the hemolymph would reach the site of cuticle synthesis. This question can only be answered empirically.

The attractive possibility of genetic analysis has not yet been fully exploited in the study of phenol oxidase. Genetic analysis of biochemical mechanisms in Drosophila is frequently impeded by the difficulty in relating biochemical function to easily screened mutant phenotypes. Phenol oxidase may represent the fortunate case of an enzyme whose modification affects external structures. It would be of particular utility to isolate strains with altered A components. The brief survey of mutant strains reported in the present work failed to reveal any electrophoretic variants of the A components. This survey could be easily extended to include many additional strains which have cuticular abnormalities. The strain alpha isolated by Lewis and Lewis bears further investigation (127, 128). They proposed that the alpha gene affects the primary structure of phenol oxidase since the enzyme prepared from this strain was more thermolabile than that from wild type. The alpha strain could be cultured on a mass scale and used as a source for the purification of A components. Lewis' and Lewis' claim

could then be verified and related to a specific protein component. Of course, the possibility discussed above, that the minor form of A_1 found in wild type is due to genetic variation should be tested. A search of wild type strains might well reveal electrophoretic variants of the A components.

The regulatory mechanisms available for the control of cuticular hardening and darkening are quite complex. Two main levels of control can be perceived, the substrate level and the enzyme level. At the enzyme level, regulation of the A components may be achieved through control of their synthesis, transport, and activation. The quantity and quality of the active enzyme would depend on the absolute and relative amounts of the various A components. An enzyme rich in A_1 would have a high specific activity on monophenols while an enzyme rich in A_2 and A_3 would act chiefly on diphenols. Analysis of genetic mosaics has shown that the epidermal cells underlying a given patch of cuticle regulate the pigmentation of that region of the cuticle (152). One way this may be accomplished is through the secretion of specific quantities of each A component into the cuticle. Regulation of the activating factors is clearly a key step in the control of phenol oxidase activity. Here, the critical reaction seems to be the activation of Pre-S. Further understanding of phenol oxidase regulation depends on the study of Pre-S activation. The tissue localization

of S-activator and active S must be known before a detailed description of the *in vivo* activation process will be possible. At the present time, it appears that synthesis or release of a small quantity of S-activator could trigger the entire phenol oxidase activation process. An analogy may be drawn with blood clotting which is the result of a cascade of limited proteolytic steps (150). Thus, phenol oxidase activation may be conceived of as the output of a biochemical amplifier. The gain of this amplifier may be sufficiently large to present serious technical obstacles to the analysis of the input stage. The amplification of minute chemical signals may be common in development, and experimental study of such phenomena may be difficult.

At the substrate level, the qualitative nature of the substrate, its amount and time of synthesis certainly influence the course of hardening and darkening. Here again the epidermal cells are important, probably by directing the pathway of substrate metabolism. For example, the N-acetylation of dopamine would favor sclerotization over pigmentation. The details of phenolic substrate metabolism must be clarified before the regulation of hardening and darkening can be understood. It is likely that hormonal factors are involved at both the enzyme and substrate levels of regulation. The facility of both genetic and biochemical analysis in *Drosophila* provides excellent opportunities for further elucidation of the developmental control of cuticle synthesis.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
DEAE	diethylaminoethyl
DOPA	dihydroxyphenylalanine
EDTA	ethylenediamine tetracetic acid
O.D.	optical density
SDS	sodium dodecyl sulfate
SE	sulfoethyl
Tris	tris (hydroxymethyl) aminomethane
u	units