STUDIES ON *DROSOPHILA* PHENOL OXIDASE

Thesis by

Alan B. Blumenthal

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1971

(Submitted February 11, 1971)

This thesis is dedicated to my parents, who encouraged me to learn, and to Dr. Tom Cheng, who introduced me to the excitement of research.

ACKNOWLEDGEMENTS

Graduate education sometimes proceeds by slow and devious ways. I am indebted to the Biology Division and many of its members for their patience and encouragement in helping me to find these ways.

My special thanks go to Dr. Herschel K. Mitchell for his gentle and perceptive guidance of my thesis work, his constant encouragement to continue the process of becoming an independent researcher, and his kindness and interest. By providing a laboratory atmosphere of friendly exchange, an example of sound experimental design, and the freedom to make lots of mistakes, he has given me an invaluable education.

There are many other people whose friendship and assistance have made my work at Caltech possible. I want to thank, in particular, a few of these people:

Drs. Bob Sanders and Ray Owen, for their continual encouragement and faith in me.

Reni Alvarez, for her friendship, cheerful company, and kindness.

Dr. Seymour Benzer, for his generosity in helping me when I most needed help.

Bill Goodell, for his constant friendship and willingness to listen.

Jon King, for showing me that you can live in Pasadena.

Sandra Winicur, for her friendship, great conversations, and insights into the Jewish mind.

Paul Meltzer, for his perceptive scientific criticism.

Jack Geltosky, for performing the tyrosine assays.

Lee Hood, for introducing me, with his characteristic enthusiasm, to research at Caltech.

Dr. Max Delbrück, for his inspiring intellectual example.

I must make special mention of Prufrock House, which was home for many years and the source of good times, companionship, and memorable dinner table conversations. The unique atmosphere of the house reflected the diverse personalities of those who lived there with me (Al Lyon, Jon King, Bill Goodell, Charlie David, Al Jesaitis, and Paul Meltzer) and those who were often there (Jeff Flatgaard, John Sedat, and Jack Geltosky). My best memories of those years will certainly be of Prufrock House.

My life at Caltech contains parts of many other lives. The one I am most thankful for is that of my wife, Donna, whose love and encouragement has been the source of energy for the completion of this thesis.

I would also like to thank the U.S. Public Health Service (Training Grant GM 86-12) for financial support.

ABSTRACT

Phenol oxidase is the enzyme responsible for hardening and pigmentation of insect cuticle. The cuticular phenol oxidase is not extractable, thus unavailable for biochemical studies. The properties and composition of the enzyme, produced *in vitro* from extracts of late third instar *Drosophila* larvae, were investigated to elucidate its *in vivo* function.

Conditions for *in vitro* activation and isolation of the enzyme were studied in detail. The conditions of activation affect both the aggregation state and the density of the resulting phenol oxidase. The enzyme was isolated from the activation extract by centrifugation on sucrose gradients.

The active phenol oxidase is an insoluble, lipoprotein complex which can be partially separated into a tyrosinase and a dihydroxy phenol oxidase. The complex is largely dissociated by strong protein denaturing agents, though some covalent cross linking is indicated. Analysis of the dissociated complex by SDS acrylamide gel electrophoresis shows a large number of protein bands. The significance of these bands is discussed in relation to the purity of the phenol oxidase and expected subunit composition.

The effects of denaturing agents, urea and sodium deoxycholate, on the phenol oxidase indicate a complex relationship between enzyme structure and activity. A tentative structural model for the phenol oxidase complex is

proposed.

The characteristics of *in vitro* phenol oxidase are discussed with regard to possible regulation of *in vivo* function.

CONTENTS

Part	Title	Page
1.	Introduction and Review	1
	A. General	1
	B. Mammalian Tyrosinase	4
	C. Insect Phenol Oxidases	8
	D. Research Objectives and Plan	19
2.	Materials and Methods	21
3.	Results	40
	Section I. Activation of Phenol Oxidase	40
	A. Activation Conditions	40
	B. Mechanism of Activation	50
	C. Substrates	56
	D. Hydroxylapetite Chromatography	58
	Section II. Purification of Phenol Oxidase	60
	A. Sucrose Gradient Centrifugation	60
	B. Purity of Phenol Oxidases from Sucrose Gradients	76
	Section III. Properties of Phenol Oxidase	79
	A. Aggregation	79
	B. Dissociation	85
	C. Chemical Composition	95
	D. Substrate Specificity	97
	E. Activity and Denaturing Agents	103
4.	Discussion	107

Part	Title	Page
A. Results		107
B. General		116
References		126
List of Abbreviations		131

1. INTRODUCTION AND REVIEW

A. General

Phenol oxidase (o-diphenol : O_2 oxidoreductase) catalyzes the ortho hydroxylation of monophenols and produces quinones from dihydroxy phenols. The reactions of this enzyme, also called tyrosinase or phenolase, have been reviewed by Mason (1). The phenol oxidase functions which have received the most attention are melanization in animals, cuticular hardening and pigmentation in insects, and browning reactions produced by injury in plants. The enzyme may also be involved in many other oxidations of phenolic compounds including the production of neurogenic catecholamines in animals and of polyphenols in plants.

All phenol oxidases contain copper at the active site and reduce molecular oxygen during catalysis. A mechanism of tyrosinase action has been proposed by Mason (2) and discussed more recently by Frieden et. al. (3). Two copper atoms in the reduced state (Cu(I)) at the active site first complex with an oxygen molecule resulting in a two electron transfer from copper to oxygen. The enzyme bound Cu_2^{+2} - $(OOH)^{-1}$ complex then transfers a hydroxyl group to a monophenol to produce a diphenol. The enzyme bound copper with one oxygen atom bound can now accept 2 electrons from a reducing agent to form water plus the original enzyme-Cu(I)

complex. The reducing agent in this case is usually a diphenol which is oxidized to a quinone. Thus, one enzyme has the dual function of a monophenol hydroxylase and a diphenol oxidase. These ideas are based on studies of plant enzymes and may not apply directly to animal phenol oxidases.

The plant phenol oxidases seem to be less complex than corresponding animal enzymes in functional organization and structure. The functions of these enzymes are probably to provide phenols for synthesis of melanin, lignin, flavinoids, tannins, and alkaloids and for hardening of seed pods, bark, etc. (1). The phenol oxidases from mushrooms (4) and *Neurospora* (5) have been well described. They are soluble proteins of molecular weight from 30,000 to 128,000, with one copper molecule per 30,000. The higher molecular weights may be due to aggregation of smaller units. Mason has proposed that these enzymes may be composed of two different subunits, each contributing a copper to the active site. Purification often greatly reduces the hydroxylase function while not affecting the diphenol oxidase. This suggests that some easily induced change in the molecule can destroy the hydroxylase while still maintaining the two copper atoms at the active site for the oxidase function (2). Perhaps there is an exchange of subunits from 1 oxidase plus 1 hydroxylase (active) to 2 oxidases (active) and 2 hydroxylases (inactive).

Mammalian and insect phenol oxidases are components of large insoluble structures and may depend on this multimolecular state for their activity. The following discussions of phenol oxidases from mouse cells and insects will point out the complexity of each system and establish a background for the experimental section of this thesis, concerning the nature of *Drosophila* phenol oxidase.

B. Mammalian Tyrosinase

A major function of tyrosinase in mammals is to produce melanin for pigmentation of skin and hair. The process of melanization has been well described cytologically and genetically, especially in the mouse (6), but the characterization of mammalian tyrosinase is less advanced.

Tyrosinase is synthesized in pigment cells or melanocytes which are found primarily in skin and retina. The melanocyte produces mitochondrial sized organelles, melanosomes, in which tyrosinase is deposited and melanization occurs. The melanosomes are retained in these cells or transferred to keratin synthesizing cells for incorporation into growing hair fibers. Moyer (7) has described the ultrastructural development of melanosomes in retinal and hair follicle melanocytes of the mouse. The melanosome is first observed as a loose network of protein containing fibers which aggregate and cross link into a matrix of parallel fibers forming a folded sheet structure. This is contained in a bilayer membrane sac. Tyrosinase molecules, which appear to be spaced regularly along the matrix fibers catalyze the synthesis of melanin until the melanosome is completely filled with the pigment. There is some evidence that the enzyme is inactivated by melanin accumulation (8) but this may be experimentally reversible (9).

Comparison of melanocyte development in a few of the many mouse coat color mutants indicates that at least three genes are involved in producing the melanosome structure,

including the tyrosinase. In retinal melanocytes mutation of the B (black/brown), C (albino), and P (pink-eyed dilution) loci seem to affect melanosome structure (7). The brown mutation (b) affects the granular quality of melanin produced and its color (brown or black). Alleles of the C locus regulate the amount of tyrosinase activity, with none present in an extreme allele (cc), but produce a normal fiber matrix. The pp genotype results in a disordered fiber matrix and faulty cross linking.

A separate study by Rittenhouse (10) of hair bulb melanocytes confirms the involvement of these three loci, with some variations. The b allele not only creates coarse, brown melanin but also a disordered fiber matrix. pp melanosomes are small and lightly melanized but normally organized. These differences illustrate the striking changes in gene expression dictated by the particular role of the melanocyte or its cell environment. They also suggest that while the C, B and P loci are involved in melanosome formation, their roles are not unambiguously defined from these studies. It is evident, however, that melanosome function and organization are closely interrelated.

Though these studies imply the presence of several melanosome proteins, a direct analysis of melanosome composition has not yet been obtained. Melanosomes have been isolated from extracts of mouse melanotic tumors and skin samples by sucrose gradient centrifugation. These are

low in density (1.245 to 1.324 g/ml) indicating a significant lipid component. They retain the ability to incorporate ^{14}C-tyrosine into melanin (7). Melanosomes are insoluble but can be dispersed by 6 M guanidine hydrochloride or 0.26% sodium deoxycholate. Most of this, however, can be centrifuged out of solution indicating the persistence of large molecular aggregates. These studies might be aided by the use of an albino cell line to eliminate melanization and possibly leave the melanosome proteins in a more soluble form.

Other studies of mouse tyrosinase have employed homogenates of melanocytes without prior isolation of the melanosomes, which were present in all stages of melanization. Menon and Haberman (9) succeeded in solubilizing the bound tyrosinase of melanoma homogenates by sonication or treatment with crude pancreatic lipase. Burnett _et al_.(11) were able to extract two soluble tyrosinases (T^1 and T^2) from the same melanoma lines (B-16 and Harding-Passey). These easily extractable enzymes were soluble at 100,000 x g and separated by acrylamide gel electrophoresis. However, part of the tyrosinase (T^3) sedimented at 100,000 x g and could be solubilized by detergents or 8 M urea. Urea yielded an enzyme with the electrophoretic mobility of T^1 while a detergent produced multiple bands of low mobility on acrylamide gels. T^1 and T^2 had similar molecular weights of about 32,000 and could be obtained from washed melanosomes.

The implication of this study is that T^1 and T^2 are precursors of T^3 which is bound to the melanosome matrix. This conclusion must await better evidence on the location of T^3 tyrosinase.

Another study (12) of soluble tyrosinases from mouse hair bulbs gave three electrophoretic forms on acrylamide gels (visualized by DOPA melanin formation). These varied in amount and occurence with the stage of hair follicle development and with mutations affecting coat color. However, there was no evidence that these enzymes were involved in melanosome formation or function.

The role of tyrosinase in relation to other proposed melanosome proteins is not clear. The enzyme may be a structural constituent of the melanosome matrix or added on to a pre-formed matrix of structural proteins. It is possible that the tyrosinase molecule is synthesized in a low molecular weight form, though the genetic studies of melanosome structure indicate that its activity is greatly influenced by melanosome organization (6,10).

C. Insect Phenol Oxidases

1. General. The role of phenol oxidases in the pigmentation and hardening of insect exoskeleton structures has been studied in many species and reviewed several times (13,14,15,16). Mitchell (17) has suggested that these enzymes may also function in the production of phenolic neurotransmitters, like adrenalin, and in the cross linking of resilin, the elastic protein used for the wing hinges and tendons in insect cuticle (18).

The discovery of the molecular nature of these enzymes has been complicated by several properties: latent activity, insolubility, and self-inactivation. Phenol oxidase activity is latent in the organism and appears only after disruption of insect tissues either by wounding, homogenization or certain chemical treatments. Once the enzyme becomes active it is insoluble and may destroy itself through oxidation and cross linking by the quinones it produces. The result is that there is no convincing published account of the nature of a purified phenol oxidase, whether active or inactive. However, the function and enzymology of several insect phenol oxidases have been analyzed and there seem to be some similarities among species. The following sections will summarize these findings with special emphasis on studies of *Drosophila*.

2. Cuticular functions of phenol oxidase. Cuticle formation in insects is a specialized property of epidermal

cells. These cells form in <u>Drosophila</u> similar cuticular structures comprising the exoskeleton, the tracheal tubes and air sacs, ducts opening to the outside of the animal, and sacs covering some internal organs. The general structure of cuticle and the process of its production has been described by Locke (19) and Mitchell (20). In brief, the cuticle is composed of dense layers of chitin and protein (endocuticle) overlying epidermal cells which secrete the cuticle and separate it, morphologically and functionally, from the hemolymph. Outside this chitinous layer is a thin proteinaceous layer termed the epicuticle or cuticulin layer. The epicuticle contains the phenol oxidase activity which is responsible for hardening and tanning of cuticular structures. The chitin layers contribute very little of the cuticle's rigidity. Larval cuticles of <u>Drosophila</u> are soft and flexible while pupal and adult cuticles are hard and often pigmented. The phenol oxidase is probably deposited in the epicuticle during the synthesis of this structure by the epidermal cells (20,21,22) and at some later time a phenolic substrate is released into the cuticle (20,23). It is also possible that some non-substrate molecules might also enter the cuticle to produce phenol oxidase activity or to modify its activity (24).

The substrate is oxidized to quinones which can polymerize and cross link the cuticular proteins, perhaps the enzyme itself. This process, known as sclerotization, has been

investigated in several insects and the mechanism discussed in detail (1,15,25). The quinone ring can react with free amino, imino and sulfhydryl groups and with other phenols to produce a tightly cross linked structure. This process also results in a change in color from pale white to tan or brown, as seen in _Drosophila_ pupal cases. In many cases (e.g. the puparium of _Sarcophaga_ or adult _Drosophila_ cuticle) the tanning process is followed by the production of black melanin, which is a polymer of indole quinones derived from tyrosine. Various authors have argued that sclerotization and melanization are separate processes which utilize different enzymes (see 13 and 16 for reviews) but the evidence is not compelling. The amount and type of substrate supplied to the phenol oxidase by underlying epidermal cells probably determines the degree of tanning or melanin formation. It is also possible that other small molecules may enter the cuticle to modify the reactivity of the phenol oxidase.

Though phenol oxidase must be present, it is not readily solubilized from white (non-sclerotized) _Drosophila_ puparia. Yamazaki has reported the extraction of an insoluble cuticular phenol oxidase from acetone powders of white puparia of _Drosophila virilis_ (26). These results, based on low enzyme activities, could be explained by contamination with non-cuticular phenol oxidase activity, which is very high at this time. Hackman and Goldberg (27)

extracted a soluble phenol oxidase from blowfly and housefly larval cuticles just before puparium formation. This could be the same enzyme involved in cuticular wound healing in these larvae (28) or possibly a contaminating trace of hemolymph enzyme. White cockroach cuticle also yielded a soluble phenol oxidase which was localized histochemically in the exocuticle,or outer laters of endocuticle (21). A similar histochemical localization was shown in the crayfish cuticle. Others have been able to demonstrate, histologically, phenol oxidase activity in various cuticle preparations. In Drosophila larvae cuticular phenol oxidase activity is easily demonstrated by the rapid blackening of the cut end of a tracheal tube place in a solution of dihydroxy phenylalanine. These results are all consistent with the function of phenol oxidase as a structural component of insect cuticle. There is still no direct evidence on the origin of this enzyme and its relationship to other phenol oxidases found in insect tissues.

3. Hemolymph and cellular phenol oxidases. Homogenates of larval, pupal, or adult Drosophila produce phenol oxidase activity after an activation period (29,30, 31). These preparations contain both hemolymph and tissue proteins. Isolated hemolymph cells also contain a latent phenol oxidase activity in Drosophila (32) and Periplaneta (21) which differs from hemolymph phenol oxidase in requiring no exogenous activating substance. The hemolymph phenol oxidase requires an additional component (activator) which

can be extracted from larval cells (33,34,35). When larvae are injured blackening occurs rapidly at the wound. This may be due to activation of hemocyte or hemolymph phenol oxidase. Substrate is available, in high concentrations, in the hemolymph.

The maximum potential phenol oxidase activity in Drosophila extracts remains at a low level until mid third instar when it rises rapidly to a peak a few hours before puparium formation. It drops rapidly after puparium formation but returns to a high level about 10 hours later at pupation. The potential activity drops slightly about 70 hours later when pigmentation of the adult cuticle begins (30). The high level of enzyme activity just before puparium formation suggests that it is used in producing this structure. However, studies of cuticle structure and synthesis (see above) indicate that the puparial phenol oxidase is placed in the cuticle many hours before this activity peak appears. Mitchell has suggested that this potential activity is used later in adult cuticle hardening and pigmentation (20).

Though direct evidence on the relationship between latent tissue and cuticular phenol oxidases is lacking, some indirect evidence has been gathered using Drosophila body color mutants (36). The prepupal activity peak is greater in blond, ebony, and wild genotypes than in yellow or black. There seems, then, to be no correlation between this

potential activity and adult body or pupal case color. The latter is true since dark adult body color is correlated with a pale pupal case (37). If phenol oxidase potential is followed through the pupal stage, its variations can be partly explained by utilization of the enzyme in adult body pigmentation (36).

The genetic analysis possible with Drosophila cannot be applied on the cytological level, as in the mouse (see above), because the epicuticle and sclerotin are amorphous structures. Mutants may be useful in elucidating the production of phenol oxidase and the dynamics of its utilization, as in the preceeding case. Lewis and Lewis (31) have isolated several Drosophila mutants which affect the level of potential phenol oxidase activity. They cannot yet be interpreted in terms of their effect on enzyme biochemistry or function. After further characterization of purified phenol oxidase and its precursors is attained, genetic analysis may be more fruitful.

4. Phenol oxidase activation and precursors. Freshly prepared extracts of Drosophila, at any stage in their life cycle, show no phenol oxidase activity. The latent activity appears after a variable lag period and increases rapidly to a plateau level, producing an S-shaped activation curve. Activation has been discribed several times in Drosophila (29,30,31,33) and in other insects: Calliphora (27,38), Marmoniella (39), Bombyx (35), and Musca (40).

Kinetic analysis of activation has shown that the rate-limiting process is an autocatalytic reaction (29). There is also indirect evidence that a protease is involved in the activation process (see below). Crude extracts contain an inhibitor of activation which can be removed by ammonium sulfate fractionation. High salt concentrations and dilution reversibly inhibit activation, but phenol oxidase substrates do not significantly affect the process.

Extracts of whole larvae contain all the needed components for phenol oxidase activation. Ohnishi (33) first observed that the hemolymph contained one part of this system and a tissue homogenate another. Several insects contain hemolymph component (s), usually called pre-phenol oxidase, which can be activated by a water soluble extract of cuticle or epidermal cells, called the activator (24,27,35,40). In Drosophila and Calliphora the activator is usually found in epidermal cells (41). The activator is so named because, in some cases, it appears to have a catalytic function. It may only affect the rate of activation and not the final level of enzyme activity. There is still not adequate evidence to confirm this possibility and some contradictory data exist (compare 24 and 38, 29 and 34).

Since the early work by Bodine and Allen (see review in ref. 13) on the phenol oxidase of grasshopper eggs, a lipid fraction has been implicated in activation. The grasshopper enzyme was activated not only by a lipid extract,

but by heat, detergents, and denaturing agents. A similar lipid effect has been suggested for the Drosophila enzyme (31). Calliphora and Musca pre-phenol oxidases are activated by detergents (27) and Calliphora, Drosophila, and Leucophaea enzymes can be activated by alpha-chymotrypsin (41,42,43). In these cases the active enzyme has a reduced substrate range and lower activity. The denaturing agents and protease must expose some site(s) necessary for enzyme activity. The lipid may interact with the pre-enzyme with a similar non-specific effect. Enzymes prepared in this way may be quite unlike phenol oxidase resulting from the natural activation process.

Further study of Drosophila phenol oxidase precursors (44) has shown that extracts can be separated by ammonium sulfate fractionation into at least 5 components : A_1, A_2, A_3, S, and P. When A components are reacted on an acrylamide gel with S and P three bands of phenol oxidase activity can be detected. All three use DOPA as a substrate for melanin formation but only the A_1 product can utilize tyrosine. S is located, in the late third instar larvae, in the salivary gland and the other components in the hemolymph (34). S is present in fresh extracts in a latent form, pre-S, which undergoes activation before participating in phenol oxidase production. S concentration or S production is probably rate limiting in the phenol oxidase activation.

Recent studies with purified S, A_1, and A_2 have shown

that these three components are necessary and sufficient to produce phenol oxidase activity (45). However, the active enzyme in homogenates or cuticle may have a different composition. It may lack S, if this has only a catalytic role, or may contain additional structural or enzymatic components.

5. Active phenol oxidase. Active phenol oxidase is insoluble and aggregates into large polydisperse particles. The enzyme has a low density (less than 1.3 g/cc), probably due to lipid components. Mitchell has investigated the aggregation of phenol oxidase in several Drosophila body color mutants and found seven density bands on equilibrium centrifugation in sucrose. The banding patterns are specific to a mutant and developmental stage. The author interpreted the density bands as a result of different proportions of precursor components in the extracts, leading to enzyme aggregates of different densities. The insoluble nature of phenol oxidase is compatible with its use as a cuticular component. The lack of phenol oxidase activity in fresh extracts of larvae and flies and the harmful effects of active phenol oxidase when injected into larvae (16) have led to the hypothesis that soluble components are brought together and activated only in the cuticle, or other sites of use, and quickly inactivated through use (36).

Because of their insolubility, insect phenol oxidases have not been successfully purified or characterized.

Karlson and Liebau (46) reported the purification of a Calliphora diphenol oxidase but their evidence for this is weak. They extracted the enzyme from an acetone powder of Calliphora larvae which probably results in a very low recovery of potential activity, as in Drosophila. Their enzyme was purified by preparative velocity sedimentation and was then shown to be pure by giving a single peak on analytical velocity sedimentation. Their other undocumented claim for purity was based on paper electrophoresis, which gives very low resolution. From the sedimentation constant they estimate a molecular weight of 530,000.

Later, Karlson, et al., (24) reported the purification of a pre-phenol oxidase and an activator from fresh extracts of larval hemolymph and epidermis, respectively. Again, evidence for purity was questionable. If this system is analogous to that in Drosophila, their activator is equivalent to S and pre-enzyme to A_1, which is more soluble in ammonium sulfate than A_2 or A_3.

6. Substrate specificity. Substrate specificity of phenol oxidases has been discussed by many authors (review in ref. 13). The ability of phenol oxidase to oxidize various mono- and dihydroxy phenols seems to depend on the source of the enzyme and the method of preparation and activation. As noted above, the activation by alpha-chymotrypin or treatment with organic solvents changes the substrate specificity of the phenol oxidase. In Drosophila,

crude phenol oxidase preparations oxidize tyrosine at about one-tenth the rate of DOPA. There does seem to be a component with almost no activity with tyrosine (36). In *Calliphora* the cuticular enzyme acts on both tyrosine and DOPA (27) but the enzyme isolated by Karlson has only diphenol oxidase activity (24,26). When added to mitochondria it also exhibits tyrosinase activity. Yamazaki (26) claims no tyrosine oxidation by a cuticular *Drosophila* enzyme, but his data contradict this conclusion.

Natural tanning agents have been investigated in several cases (see 1,14) and in *Calliphora* it appears to be n-acetyl dopamine (47). The tyrosine side chain is decarboxylated and acetylated to form this compound. For tanning of the cockroach egg case tyrosine is further degraded to dihydroxybenzoic acid (14). The formation of melanin *in vitro* seems to require the cyclization of the tyrosine side chain to form an indole (1). Insect phenol-oxidases can also utilize tyrosine or DOPA *in vivo* to form indole melanins (48).

Both yellow and black melanin occur in *Drosophila* and other insects. Experiments on pigmentation of cricket cuticle (49) indicate that both pigments are due to the oxidation of tyrosine derivatives. The same author has shown that cuticular phenol oxidase can utilize Dopa and tyramine (but not tyrosine) to form black pigments, while N-acetylated tyramine produces a strong yellow pigment.

D. Research Objectives and Plan

The object of the experimental work described in this thesis was to determine the chemical and subunit composition of active phenol oxidase from *Drosophila melanogaster*. *In vitro* phenol oxidase was used, rather than the cuticular enzyme, because it is easily available. All of the studies on *Drosophila* cuticular enzyme have demonstrated the futility of attempts to isolate this phenol oxidase. The major disadvantage in using *in vitro* phenol oxidase is the lack of direct evidence on its relationship to the cuticular enzyme. Until this can be established, however, data obtained with the *in vitro* enzyme may help to set general limitations on the properties and function of the enzyme *in vivo*, especially in the cuticle.

By dissociating the active and aggregated enzyme it was hoped to answer these questions: (1) What is the subunit composition of active phenol oxidase? (2) How do different density forms of active phenol oxidase differ in composition? (3) What is the nature of the intermolecular bonds in the active enzyme? and (4) What is the average chemical composition of purified phenol oxidase? These objectives have been only partially realized due to difficulties in purifying and dissociating the enzyme.

The *ebony* mutant, which has dark adult body pigmentation, was selected to begin these studies because it is

easy to culture (high viability); it yields high phenol oxidase activity at puparium formation; and most of its enzyme activity can be easily isolated from sucrose equilibrium gradients.

During the course of this work it was necessary to re-investigate the conditions for activation and purification of phenol oxidase. These results are present in sections I and II. Attempts to degrade the phenol oxidase were only partially successful. These results, along with some biochemical characterization of active enzyme, are given in section III.

2. MATERIALS AND METHODS

1. Culture and collection of Drosophila.

Drosophila melanogaster of the ebony (e") or Oregon R wild type were raised as described by Mitchell and Mitchell (50). Eggs were collected for 1½ to 2½ hours, depending upon the rate of egg laying. Larvae were collected from the sides of the leucite collecting boxes when at least 10 to 20% had formed white puparia but few tan puparia were present. For some experiments this mixture of late third instar larvae and white prepupae were washed in water (25°C), partly dried on filter paper, and used immediately for preparation of phenol oxidase. In most cases, however, they were washed, dried, and quickly frozen by adding liquid nitrogen to a beaker containing the animals. These were stored up to 6 months at -75°C with no apparent change in potential for phenol oxidase production.

2. Preparation of phenol oxidase.

The preparation of phenol oxidase was essentially the same as that used by Mitchell et al. (36). 10 grams of late third instar larvae and prepupae, live or frozen at -75°C, were added to 5 grams of sand and 20 ml 1.5 M KH_2PO_4 in a 600 ml mortar cooled in an ice bath. The sample was rapidly ground to a smooth paste (in about 5 min) and diluted with 130 ml of distilled water at 0°C. The homogenate was

filtered through nylon bolting cloth and centrifuged at 55,000 x g (average force) for 10 min. This and all further operations were conducted at 4 to 6°C. The supernatant was poured through nylon bolting cloth, to remove floating lipid material, and 50 ml of $(NH_4)_2SO_4$ solution, saturated at 4°C in 0.02 M phosphate buffer, pH 6.7, was slowly added with stirring to the supernatant. The volume of this solution was measured and additional $(NH_4)_2SO_4$ solution added to 56% saturation. The precipitate was collected by centrifugation at 55,000 x g for 10 min and then processed by one of the following procedures.

Procedure I. The $(NH_4)_2SO_4$ pellet was washed by resuspension in 30 ml 56% saturated $(NH_4)_2SO_4$, made up in 0.02 M phosphate buffer, pH 6.7, and centrifuged at 66,000 x g for 5 min. This pellet was dissolved in 7.5 ml of 1.5 M KCl in 0.02 M phosphate buffer, pH 6.7, and centrifuged at 59,000 x g for 20 min. The supernatant was desalted on a 40 ml bed volume of Sephadex G-25 (fine) prepared in 0.02 M phosphate buffer, pH 6.7. The excluded protein (in about 15 ml) contained the activating phenol oxidase.

Procedure II. The $(NH_4)_2SO_4$ pellet was washed by resuspending it, with a glass homogenizer, in 160 ml of 56% saturated $(NH_4)_2SO_4$, in 0.02 M phosphate buffer, pH 6.7. The precipitate was collected by centrifugation (55,000 x g) for 10 min. This step was repeated once. The precipitate,

now divided equally into 8 tubes, was stored at -75°C until needed or dissolved in 0.1 M buffer for activation. Within 10 min after resuspending the pellet in buffer for activation, the solution was centrifuged at 55,000 x g for 10 min. There was no loss of activity in this step and maximum activity was 5 to 7 % greater than an uncentrifuged control. Activation was always carried out at 0°C.

All centrifuge forces are given for the middle of the tube, unless otherwise specified.

3. Sucrose gradient centrifugation.

Several types of sucrose gradients were used in this work. They were all linear and pre-formed either in glass mixing chambers, as described by Mitchell *et al*. (36), or with a Technicon peristaltic pump to meter the mixing of the two sucrose solutions and to deliver this mixture to the centrifuge tube. The gradients were usually made from the following sets of solutions: 0.7 g and 1.8 g of sucrose per ml of buffer (densities 1.190 to 1.310 g/ml), 45% to 64% (w/v) sucrose (densities 1.182 to 1.333 g/ml), and 5 to 20% (w/v) sucrose (densities 1.029 to 1.093 g/ml), in the appropriate buffer. These were run at 4°C in the Spinco L2-65 centrifuge in the SW 25.1, SW 25.2 or SW 65 Ti rotor. Typical sample volumes were 2.5 to 3 ml, 4 to 6 ml, and 0.1 to 0.25 ml, respectively. In most cases phenol oxidase samples were applied to the gradients and centrifuged as

soon as maximum activity was reached. Gradients were centrifuged at 25,000 x g (avg. force) for 16 hours at 4°C. At the end of centrifugation the cellulose tubes were pierced at the bottom with a needle and either 0.9 or 1.9 ml fractions collected into graduated test tubes.

Active phenol oxidase, in sufficient concentration, produces opalescent bands in these gradients which can be clearly seen through the tube. Collecting fractions caused spreading of these bands. Band 2, near the bottom of the tube, is contained in about 2 ml in a 50 ml gradient but because of its sticky and flocculent characteristics it slowly drained out of the tube in 6 to 10 mls. Also, bands near the top of the tube which could be visually resolved could not be enzymatically resolved due to spreading during fractionation.

All densities of sucrose solutions were determined in duplicate by weighing samples at 4°C in a calibrated 250 micro liter Lang-Levy pipette

4. Phenol oxidase assays.

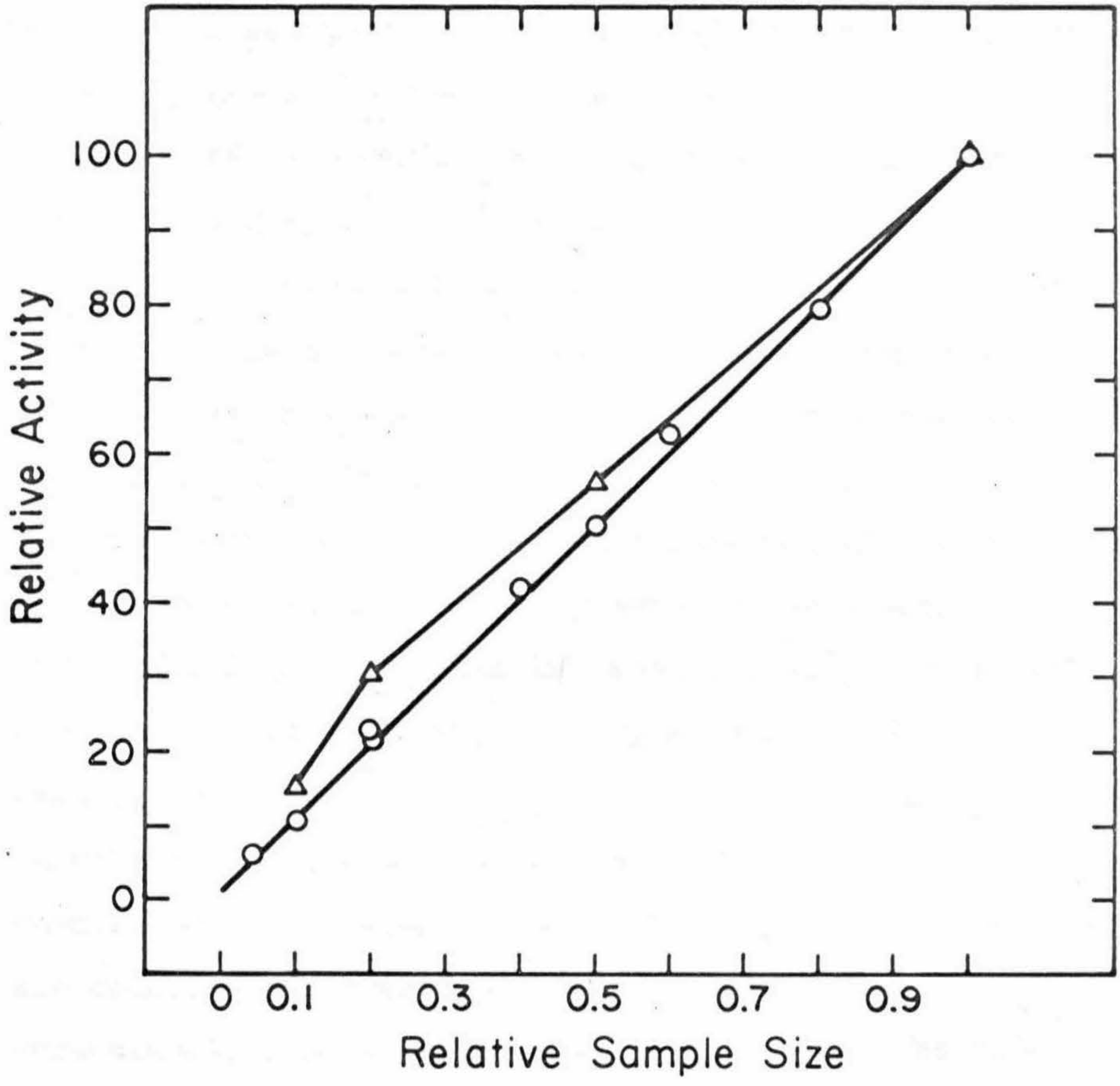
Phenol oxidase samples of 5 to 100 micro liters were added to 0.6 ml of 0.004 M DOPA solution (80 mg D,L-dihydroxy phenylanine/100 ml of buffer) in 0.02 M phosphate buffer, pH 6.3, at 30°C. The samples were measured into the substrate solution with disposable glass micro pipettes (Corning Glass Co.), rapidly mixed, and transferred to a

cuvette (1cm light path, 4 mm wide) of the Cary 11 recording spectrophotometer. The change in OD_{475} was continuously recorded for 1 to 3 min with a chart speed of 1 in/mim. During this time the increase in optical density was linear for most samples. The slope was measured and recorded as change in OD_{475}/10 min. A change of one OD unit at 475 n m in 10 min is equivalent to one unit of activity.

For a given phenol oxidase preparation, sample volume vs DOPA oxidase activity is linear over a wide range (Figure 1). At activities greater than 4 or 5 units, this curve flattens off (not shown), probably due to limiting substrate plus rapid inactivation of the enzyme by melanin formation. In Figure 1 the intercept on the ordinate (infinitely low enzyme concentration) is several percent of the maximum measured activity. This is not due to variation in total volume of the assay mixture but probably to auto-oxidation of the substrate. Unless specified, all phenol oxidase activities were determined with DOPA as the substrate.

Monophenol oxidase activity is assayed in a similar way using 0.4 mg of L-tyrosine (A grade, Calbiochem) per ml of buffer or a saturated solution at 30°C (both about 0.002 M) in 0.02 M phosphate buffer, pH 6.7. With tyrosine as a substrate there is a lag period before production of red color (dopachrome) which is inversely proportional in length

Figure 1. Phenol oxidase activity as a function of enzyme sample size. Sample size (5 to 100 microliters) and activity (up to 4 units) have been normalized to maximal values of 1 or 100, respectively. The circles represent DOPA as substrate and the triangles tyrosine.



Relative Activity
100
80
60
40
20
0
0
0.1
0.3
0.5
0.7
0.9
Relative Sample Size

to the phenol oxidase activity. The lag is shortened by addition of 10^{-4} to 10^{-3} M DOPA but, since this contributes significantly to the measured activity, it was not used for tyrosinase measurements. In a typical assay with tyrosine a lag of 20 sec to 10 min is followed by a gradual color intensity increase, then a short linear phase, a rapid leveling off, and a slow decline. It is necessary to wait for the leveling off in each assay to measure accurately the linear portion of the curve. When the rate of linear increase in color intensity is compared to sample size (Figure 1) a straight line results except at low activities (less than 0.1 units).

In many cases, activity yields of phenol oxidase from density gradients or chromatographic columns were calculated. These were highly reproducible when activity measurements were done in the same way but may vary 10 to 20% from the true activity. One major cause of error was that activity recoveries calculated from low activity values were greater than for high activity measurements. This would decrease the calculated recovery, since fractions of high activity were usually assayed without dilution. Also, the calculation of total input activity was uncertain because of a 5 to 10% change in the activity of this sample during the experiment. The direction of this error could not be estimated, for the fractionated phenol oxidase activity may not have the same stability characteristics as the starting

material. In all cases a sample of the initial enzyme solution and the fractionated material were assayed at the same time.

5. Preparation of phenol oxidase density bands.

Density bands 2 (from ebony) or 3 (wild type) of aggregated phenol oxidase were isolated from sucrose equilibrium gradients. The fractions, collected from the bottom of the centrifuge tube, containing flocculent, aggregated enzyme were pooled and diluted 5 times with buffer. In some cases sucrose was removed by diluting 10 times with buffer or distilled water and pelleting the enzyme at 21,000 x g for 30 min, at $4^{o}C$. This pellet was rinsed twice in 30 ml of distilled water by resuspension in a glass homogenizer and re-centrifugation. There was some material lost through adherence to glass surfaces. This phenol oxidase was then resuspended in a small volume of distilled water and lyophilized to dryness. The tan colored powder resulting had no phenol oxidase activity. It was stored over $CaCl_2$ and used in some dissociation experiments and for analysis of phosphorous, sugars and amino acids.

6. Protein determinations.

The Folin-Lowry (51) method for protein determination was modified to allow for the partial hydrolysis of insoluble proteins and elimination of contaminating solutes

(urea, etc.). A sample was placed in the bottom of a small tube (7.7 cm long, 0.9 cm i.d.), with a flat inside bottom, and the volume adjusted to 100 micro liters with distilled water. To this 0.4 ml of 25% (w/v) TCA was added and the precipitate centrifuged for 45 min, at 14,500 x g in the Sorvall SS-1 rotor, at room temperature. The supernatant was carefully removed with a pasteur pipette and the tubes drained thoroughly. The tubes were cooled in an ice bath and 100 micro liters of 1N NaOH added. They were heated in a boiling water bath for 10 min, then cooled rapidly in water. This time was adequate to dissolve a phenol oxidase sample, while further heating caused a greater loss in detectable protein.

One ml of the alkaline copper tartrate reagent (50 ml 2% Na_2CO_3, 0.5 ml 1% $CuSO_4$, 0.5 ml 2% Na Tartrate, mixed before each experiment) was added to each tube and incubated for 10 min at room temperature. 100 micro liters of phenol reagent (Harleco Chem.), diluted 1:1 with distilled water was added and mixed rapidly on a Vortex mixer. After 30 min the OD_{660} was determined with the Cary 15 spectrophotometer. BSA standards (crystallized, Sigma Chem. Co.) were treated in the same way and gave a linear standard curve from 5 to 80 micro grams/ml. All samples were run in duplicate.

Samples containing urea or SDS were precipitated by TCA, as above, rinsed with 1 ml of 20% (w/v) TCA, and recentrifuged. This additional step did not affect the

color yield of BSA standards. Deoxycholate could be removed with absolute ethanol, but a significant amount of protein was also lost.

7. Tyrosine assays.

The tyrosine content of phenol oxidase preparations was determined by a modification of the method of Waalkes and Udenfriend (52). These assays were performed by Mr. Jack Geltosky. Samples of 0.5 ml were mixed with an equal volume of cold 15% TCA (w/v) and kept at 0^oC for one hour or more. They were then centrifuged (8,000 x g for 20 min, 4^oC) and 100 micro liter samples of the supernatant used for tyrosine determinations.

This sample was added to 0.2 ml of a reagent made from 1.5 ml of 0.1 M $NaNO_3$, 1.5 ml of 3 N HNO_3, 1.0 ml 0.05% of nitrosonapthol, and 0.75 ml of 95% ethanol. This mixture was incubated at 55^oC for 30 min., cooled, and mixed with 2.5 ml of 25% ethanol. The fluorescent emission at 555 n m was measured in a Farrand spectrofluorometer with excitation at 465 n m. Tyrosine standards give a linear curve from 0.75 to 4.5 n moles per sample.

8. Amino acid analysis.

Lyophilized phenol oxidase from wild type animals (density band 3) or the _ebony_ mutant (density band 2) was weighed out on a torsion balance (about 1 mg) and hydrolyzed

in 1 ml of 6 N HCl in an evacuated, sealed tube at 110°C for 20 hrs. The brown hydrolysate, which contained considerable black precipitate, was evaporated to dryness. This material was run, by Mr. John Racs, on a Beckman amino acid analyzer. The peak areas were calculated from the peak height and width at half the total height.

9. Phosphate determinations.

The determination of total phosphate in lyophilized phenol oxidase was carried out as described by Ames (53). A milligram or less of protein was weighed out on a torsion balance and placed in clean test tubes washed in a phosphate-free detergent. Samples, standards, and water blanks were evaporated to dryness and washed twice in ethanolic $Mg(NO_3)_2$. All reagents were made up in glass distilled, deionized water. Optical density was determined at 670 n m with a Cary 15 spectrophotometer. A linear standard curve was obtained with 7 to 180 n moles of Na_2HPO_4. A control of 1.20 mg samples of BSA (crystallized, Sigma Chemical Co.) gave readings comparable to the water blanks.

10. Hexose determinations.

Total hexose content of lyophilized phenol oxidase was determined by the anthrone reaction as described by Ashwell (54) with the following modifications. The dry enzyme was weighed into dry, acid washed test tubes and 0.5 ml of distilled water added. After cooling, cold

anthrone reagent (anthrone from Matheson, Coleman and Bell Chemical Co.) was carefully added. Glucose was used for a standard and gave a linear curve from 4 to 70 micro grams. The optical density at 625 nm was measured with a Cary 15 spectrophotometer. A BSA control gave a color intensity equivalent to 1 microgram of glucose per mg of BSA.

11. Gas chromatography.

To determine the presence of fatty acids 3.43 mg of washed lyophilized phenol oxidase (band 2) was treated with 5 ml of petroleuom ether, 19 ml of dry methanol, and 1 ml of concentrated sulfuric acid. This was refluxed at 100°C for 4 hours to hydrolize off the fatty acids and to form methyl esters. After cooling the condenser was rinsed with 10 ml petroleum ether and this rinse added to the reflux mixture. An additional 25 ml of petroleum ether and 10 ml of distilled water were added and mixed in a separatory funnel. The petroleum ether layer was removed with a syringe and the aqueous phase twice more extracted with 35 ml each of petroleum ether. The combined petroleum ether extract was washed twice with 100 ml each of distilled water and then dried over anhydrous sodium sulfate. The dry petroleum ether was evaporated at 45 to 50°C, under reduced pressure, and the residue immediately taken up in 0.5 ml of warm petroleum ether. This was stored at -10°C.

10 ml samples were analyzed with a Loenco Model 70

Hi-Flex gas chromatograph. The columns were ¼ inch x 6 ft. stainless steel coils packed with Chromosorb W (Reg., 60/80 mesh) as the solid phase and Lac 728 (diethylene glycol succinate) as the stationary phase. Helium was the carrier gas at 25 psi input pressure. Sample column flow rate was 65.6 ml/ min and the reference column 92.3 ml/min. The following temperatures were maintained constant through the analysis: columns, 170°C; detector, 227°C; and injection ports, 300°C. A thermal conductivity detector was used at 100 ma current with an attenuation of $^{x}1$. The chart recorder speed was 0.5 in/min.

Standard methyl esters were run along with a 10 mg sample of BSA treated in the same way as the phenol oxidase. The latter gave a base line response.

12. Column chromatography.

Sephadex, Sepharose, and Biogel were prepared as recommended by their manufacturers (Pharmacia Fine Chem. and Bio-Rad Labs.). Sephadex G-25 (fine) was washed extensively in distilled water to remove fine particles, then equilibrated with buffer and stored at 4°C. It was poured into small columns made of soft glass tubing constricted at one end and plugged with glass wool. For desalting protein samples 5 bed volumes were used for a given sample volume. Other gels were equilibrated with starting buffer and poured into Sephadex or Chromoflex (Kontes Glass Co.)

chromatography columns. In the latter, the sintered plate was first covered with two layers of fine nylon gauze. When sodium deoxycholate was used, the column was first packed in buffer without detergent, then equilibrated with the buffer plus detergent. Blue Dextran (Pharmacia Fine Chem.) was used to determine the void volumes. All columns were run at 4 to 6°C and fractions were collected volumetrically.

Hydroxylapetite gel (Biogel HT) was washed free of small particles in cold 1 mM sodium phosphate buffer, pH 6.7, and packed into small glass columns plugged with glass wool. The sample was usually mixed with a slurry of gel and then packed into the column under pressure. The initial effluent was passed once through the packed bed. Protein samples were applied at 2 to 5 mg per ml of bed volume. Elution was by increasing phosphate concentration, at pH 6.7, in a linear gradient or stepwise. All elution was done with hydrostatic pressure (50 cm) or air pressure (3 to 4 lb/in^2) on the gel bed, which had a surface diameter of 1.8 cm.

13. Acrylamide gel electrophoresis.

Acrylamide gel electrophoresis in SDS was performed by the method of Weber and Osborn (55). Gels were 10% acrylamide and 0.14% bis-acrylamide (half cross linked). Gels were run at 6 ma/tube until the tracking dye had moved 9 cm. This time varied with the amount of protein in a

sample, presumably due to the amounts of precipitated protein at the top of the gel. Gels were usually stained for 24 hours in 0.25% Coomassie Brilliant Blue, R-250, (Mann Res. Lab.) in methanol:acetic acid:water, 5:1:5 by volume, for 24 hours. They were destained electrophoretically or by diffusion in 5% methanol and 10% acetic acid.

For some experiments gels were first fixed in several changes of 20% sulfosalicylic acid overnight, then stained in 0.25% Coomassie Brilliant Blue in distilled water for at least 12 hours and destained by diffusion in 10% acetic acid. The results of these two methods were identical.

Gel length and tracking dye position were measured before removing the gels from their tubes. Band positions and gel length were measured after staining. Mobilities were calculated as described in reference 55. In some cases destained gels were scanned in a Gilford spectrophotometer (wavelength:600 nm, scanning slit: 0.05 mm). This method was more sensitive than visual observation but gave slightly lower resolution of closely spaced bands.

Samples and standards were prepared by heating at 100°C for 20 min in 0.1% (w/v) SDS, 0.1% (v/v) beta-mercaptoethanol, and 0.1 M sodium phosphate, pH 7.0. Standard samples prepared in 1% SDS, 1% beta-mercaptoethanol gave the same patterns and mobilities when applied in this buffer. Standard proteins used were myoglobin (A grade, Calbiochem), ovalbumin (crystallized, Grade V, Sigma Chem. Co.), BSA

(Crystallized, Sigma Chem. Co.), catalase (C-100, Sigma Chem. Co.) and phosphorylase a (P-0253, Sigma Chem. Co.).

14. Radioactive sample preparation and scintillation counting.

Frozen E. coli cells labelled with ^{14}C-leucine (approx. 5 µc) were supplied by Dr. Robert Mayol. These were thawed, pelleted at 8,000 x g for 10 min and resuspended in 0.05 M Tris buffer, pH 8.05, 0.01 M Na_2 EDTA, and 0.01% egg white lysozyme. This mixture was incubated at 37°C for 15 min then freeze-thawed three times to disrupt the cells. This solution was then incubated with DNAase at 37°C for 30 min. The supernatant (9,300 x g, 15 min) was chromatographed on Biogel P-10 in 0.02 M phosphate buffer, pH 6.7 and the radioactive material in the excluded volume pooled and lyophilized. This material contained about 1 to 1.5 mg of protein with about 10^6 cpm of ^{14}C-leucine.

Radioactive samples were counted in a Beckman LS-200 Scintillation counter. 0.05 to 1 ml samples were added directly to 10 ml of scintillation fluid (3Kg dioxane, 300 g napthalene, 15 g PPO) and counted for 10 min at a gain of 250 in a pre-set ^{14}C window. Quenched and unquenched ^{14}C standards (Nuclear Chicago) were used to determine counting efficiency (93%) and a quench correction curve utilizing external standardization. The quenching in experimental samples was always less than 5%. Results are reported as

uncorrected cpm, less the background of 40 cpm.

When high concentrations of sucrose were present in the samples, water was added up to 1 ml to prevent sucrose precipitation in the dioxane scintillation fluid.

15. Sample concentration.

Concentration of active phenol oxidase solutions in dialysis tubing, against Sephadex gels or dry air, results in large loses of protein (up to 50%) even in the presence of SDS. Active enzyme solutions can be concentrated with no activity loss by pressure dialysis on PM-30 membranes (Diaflow Membranes, Amicon Corp.). This was done under 20 lb/in^2 of pressure for a 43 mm membrane, (Amicon Model 52 cell) or 10 lb/in^2 for a 25 mm membrane (Amicon Model 12 cell), at $4^{o}C$.

16. Reagents and buffers.

All chemicals used were reagent grade except urea (Ultra Pure, Mann Res. Lab.), sodium dodecyl sulfate (Technical, Matheson, Coleman and Bell), sodium deoxycholate (M.A., Mann Res. Lab.), diisopropyl fluorophosphate (M.A., Mann Res. Lab.), and guanidine-HCl (Ultra Pure, Mann Res. Lab.).

Phosphate buffers were a mixture of sodium and potassium salts, except for SDS acrylamide gels and hydroxylapetite chromatography. Tris buffers were made with Trizma Base

(Sigma Chem. Co.) and titrated with concentrated HCl. Measurements of pH, unless specified, were at 24°C with a Beckman No. 39030 combination electrode. In some cases the pH of a solution at 0°C was measured after standardizing the pH meter (Beckman, Expanded Scale) with a pH 7.0 standard solution at 0°C. The values obtained for tris buffers at 0°C were within 0.2 pH units of those calculated from published data (56).

3. RESULTS

Section I. Activation of Phenol Oxidase

A. Activation Conditions

The course of appearance of phenol oxidase activity in Drosophila extracts is shown in Figure 2. Parameters which will be used to describe the activation process are the lag period until measurable enzyme activity appears, the rate of rapid activity increase, the time to constant activity (or the plateau region), and the level of constant activity. The variables of protein concentration, pH, and buffer in activation were investigated to determine the conditions for maximum phenol oxidase yield. Activation conditions were also varied in an attempt to produce a more soluble form of the active enzyme.

1. Concentration. Phenol oxidase activation was monitored at several extract concentrations to determine the effect of dilution on the parameters of this process. Concentration of the extracts is given in terms of equivalent wet weight of animals extracted per ml of activation solution. Activation was more rapid, with a shorter lag period, at high extract concentrations. This is shown in Figure 2 and by curve A of Figure 3. There is a rapid increase in activation time beyond a certain dilution (0.067 g/ml). At high activation concentrations, the activation curve "overshoots" or reaches a peak of activity then drops as much as

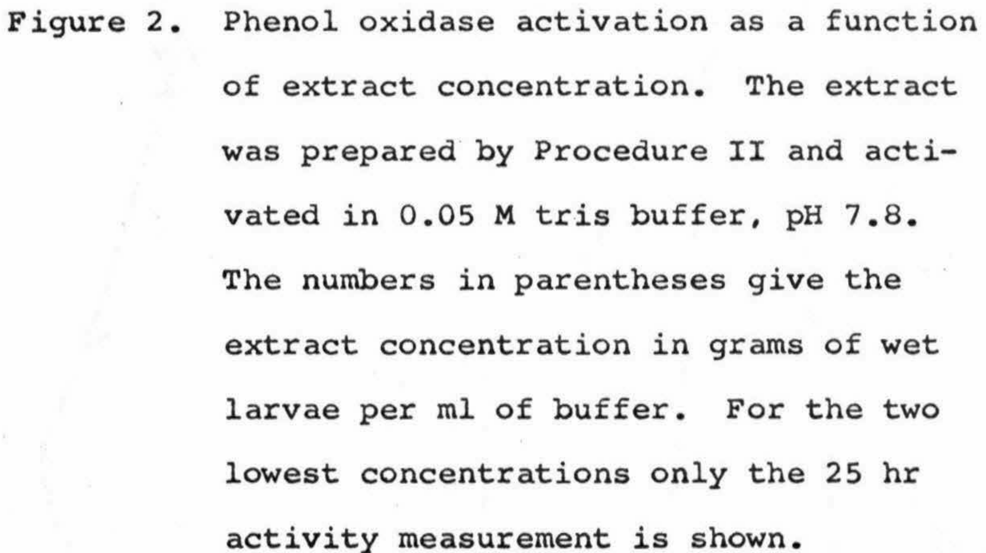
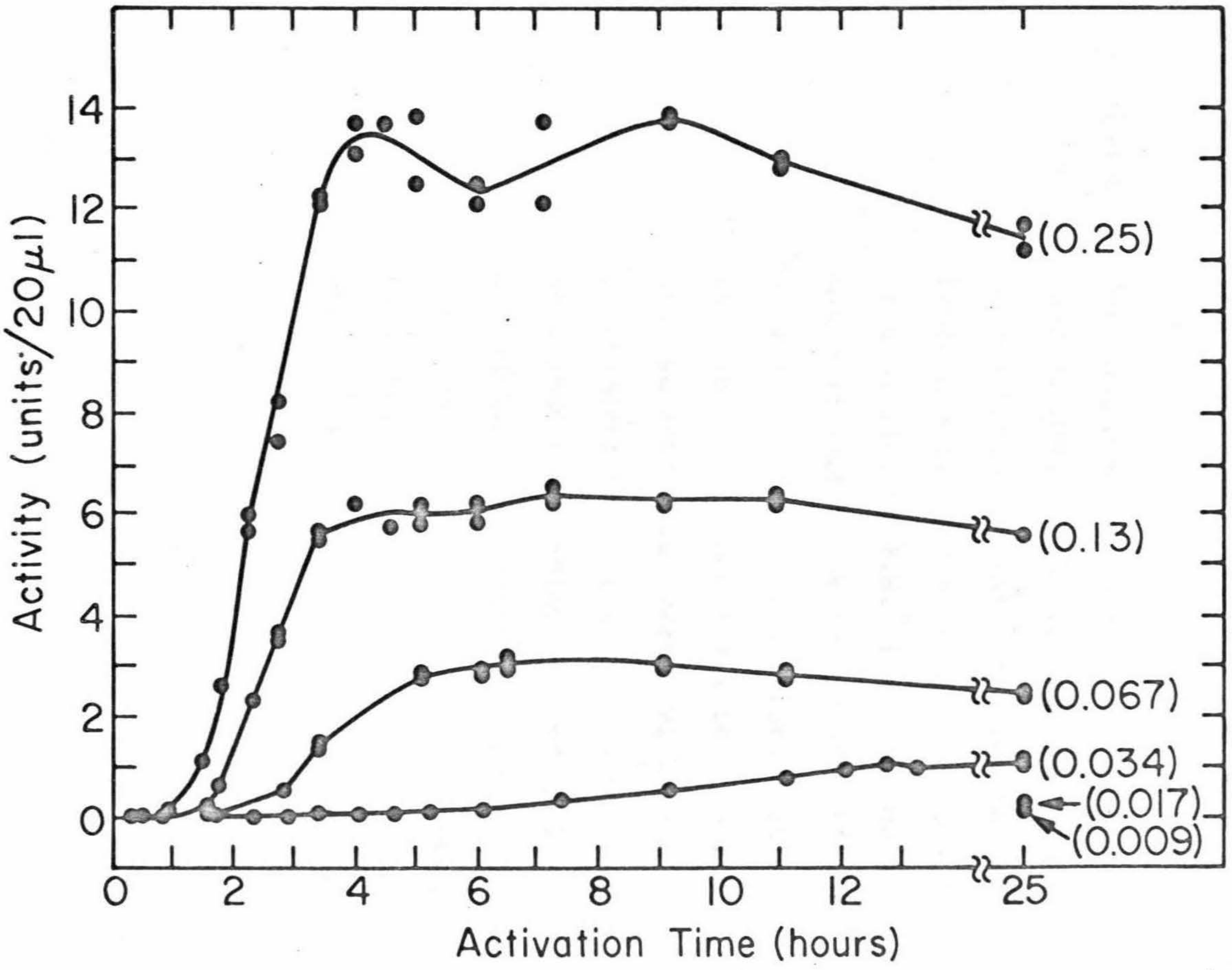
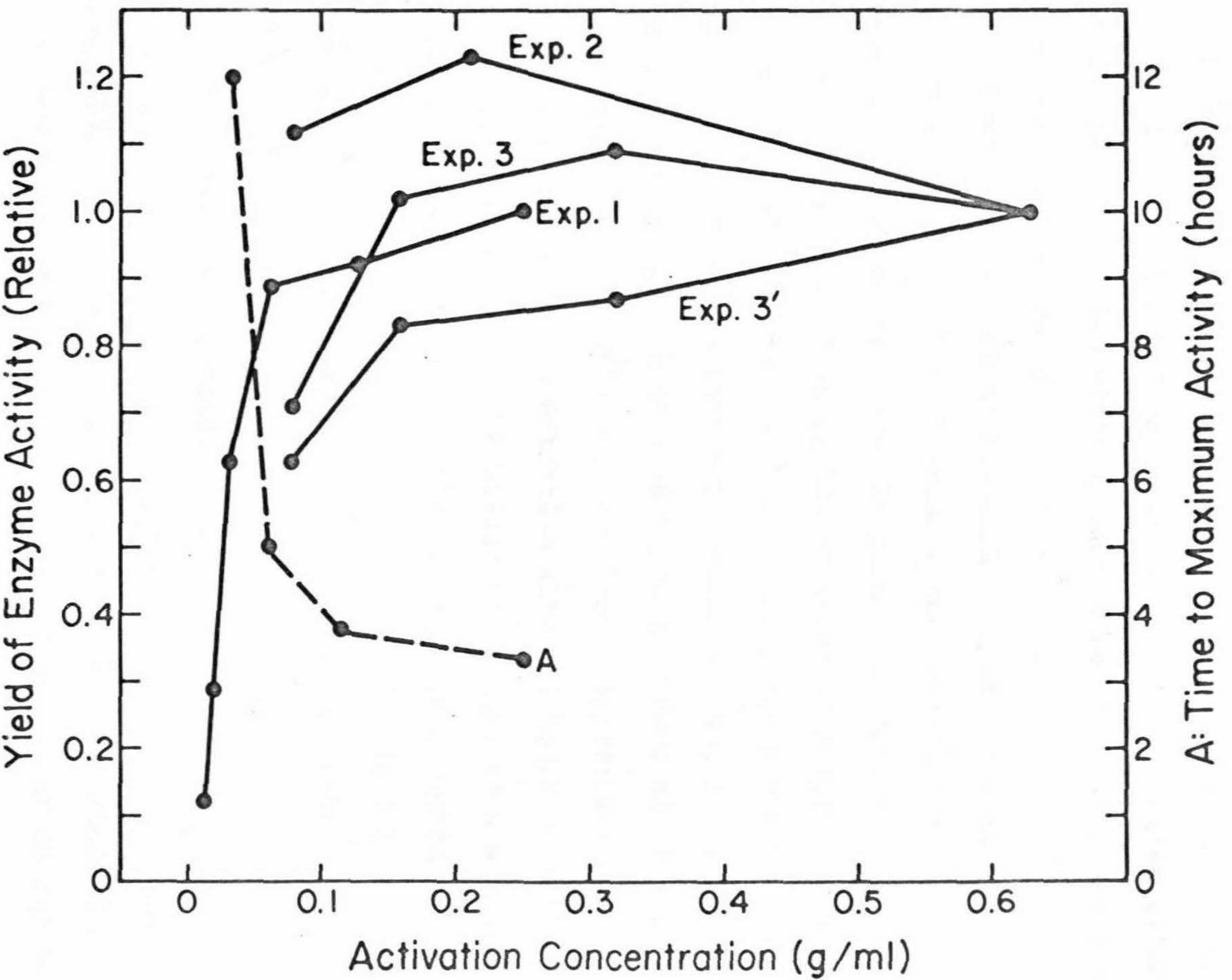


Figure 2. Phenol oxidase activation as a function of extract concentration. The extract was prepared by Procedure II and activated in 0.05 M tris buffer, pH 7.8. The numbers in parentheses give the extract concentration in grams of wet larvae per ml of buffer. For the two lowest concentrations only the 25 hr activity measurement is shown.



Activity (units/20μl)
0
2
4
6
8
10
12
14
Activation Time (hours)
0
2
4
6
8
10
12
25
(0.25)
(0.13)
(0.067)
(0.034)
(0.017)
(0.009)

Figure 3. Activation time and yield of phenol oxidase activity at various activation concentrations. Extracts prepared by Procedure II were activated in 0.05 M tris buffer, pH 7.8. Yield was determined at each point by the activity of a small sample times the total activation volume. Since absolute phenol oxidase activities varied with the experiment, the values are normalized to 1 unit of activity at 0.63 g/ml activation concentration. Time to reach maximum activity (curve A) was taken from the data of Experiment 1, which is fully represented in Fig. 2.



Exp. 2
Exp. 3
Exp. 1
Exp. 3′
A
Yield of Enzyme Activity (Relative)
A: Time to Maximum Activity (hours)
Activation Concentration (g/ml)

60% in a few hours to a plateau level. The occurrence of this overshooting is variable and may be related to the heavy precipitation which occurs in concentrated activation extracts. In the plateau region activity may increase or decrease slowly (up to 10% in 24 hours).

Activity measured at the beginning of the plateau region is linear with activation concentration for a given experiment. However, low activation concentrations give decreased phenol oxidase yield, as shown in Figure 3, when maximum activities are compared. In experiments 2 and 3 the most concentrated activation solutions (0.63 g/ml) seem to give a lower phenol oxidase yield than more dilute solutions. To test this, all samples in experiment 3 were diluted to the same concentration after 11 hours of activation and then assayed. The yields calculated show a constant decrease with dilution (Experiment 3'). The apparent low values at 0.63 g/ml may be due to errors involved in measuring high phenol oxidase activities (see: Materials and Methods).

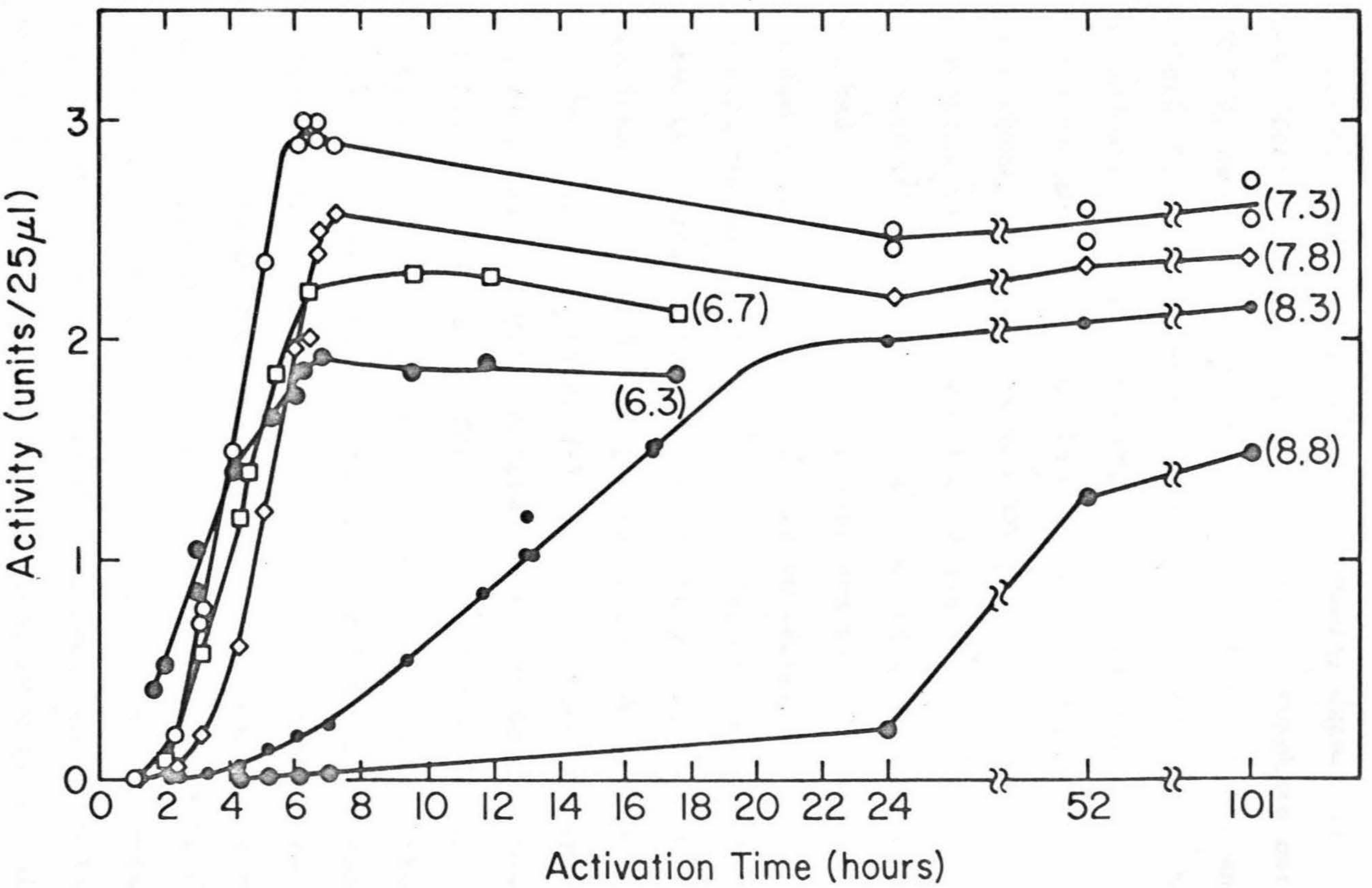
The activation concentration at which enzyme yield drops off also seems to greatly increase activation time (Fig. 3). This concentration varies with the preparation and must be determined in each case. This variation may be due to uncontrolled factors in the grinding of larvae resulting in quantitative and qualitative differences in the extract. The inhibition of activation by dilution suggests that the rate

limiting step is a bimolecular reaction. However, several steps in activation may be affected by dilution. This is consistent with the postulated complexity of the activation process which includes the conversion of pre-S to S, and the interaction of S,.A_1, and A_2 (see Introduction). It is not known whether the inhibition by dilution is completely reversible.

2. pH. To determine the effects of pH on activation, phenol oxidase was prepared according to procedure I, but desalted on Sephadex G-25 in distilled water. Samples of the enzyme were diluted immediately into equal volumes of 0.1 M buffer to give an activation extract concentration of 0.06 g/ml. The results are shown in Figure 4. In general, the lag period decreases with pH. From pH 7.3 to 8.8 (pH 8.0 to 9.6 at $0^{o}C$) maximum enzyme activity is inversely related to pH. However, pH 6.3 activity was 20% lower than at pH 6.7. A comparison of activities after 24 hours gives a broad pH optimum with a maximum between pH 6.8 and 8.3 (measured at $0^{o}C$). The rate of rise of the activation curve also has a pH optimum between these values but with a rapid drop-off at high pH and a gradual decrease at a lower pH.

3. Buffer composition and ionic strength. Preliminary observations suggesting a phosphate inhibition of

Figure 4. Activation of phenol oxidase at several pH values. Extracts prepared by procedure I were activated at a concentration of 0.06 g/ml in 0.05 M tris (pH 7.3 to 8.8) or phosphate buffer (pH 6.3 and 6.7). The numbers in parentheses are the pH values of each activation buffer, measured at 24°C.



Activity (units/25µl)
0
1
2
3
Activation Time (hours)
0
2
4
6
8
10
12
14
16
18
20
22
24
52
101
(7.3)
(7.8)
(8.3)
(8.8)
(6.7)
(6.3)

phenol oxidase activation led to several experiments on the effect of buffers on this process. Phosphate buffer, pH 6.7, was tested at concentrations of 0.1, 0.05, and 0.01 molar. The most obvious effect was an increase in the lag period with phosphate concentration. The rates of activity increase for the samples in 0.1 and 0.05 M phosphate buffer were equal, but the rate was 30% greater at 0.01 M phosphate concentration. As a result of these two factors, lag period and rate of activity increase, each of the three samples reached maximum activity an hour apart. This result could be due specifically to phosphate concentration or generally to salt concentration. High salt (1.5 M NaCl or KCl) is known to inhibit activation completely, while lower concentrations (1 M and 0.5 M) greatly delay the process.

To test these possibilities the preceeding experiment was repeated with 0.025 M tris-maleate buffer, pH 6.65, and 0.05 M phosphate buffer, pH 6.7. The pH values measured at $0^{o}C$ were 6.80 and 6.85, respectively. Half-maximum activity was reached at about 3 hours in tris maleate buffer and 4 hours in phosphate buffer. The same maximum activity was attained at about 6.5 hours in each sample. Since activation was not monitored for the first two hours, it is not possible to distinguish the effects on lag period or rate of activity increase. The results suggest, that phosphate concentration, as well as salt concentration, may effect the overall

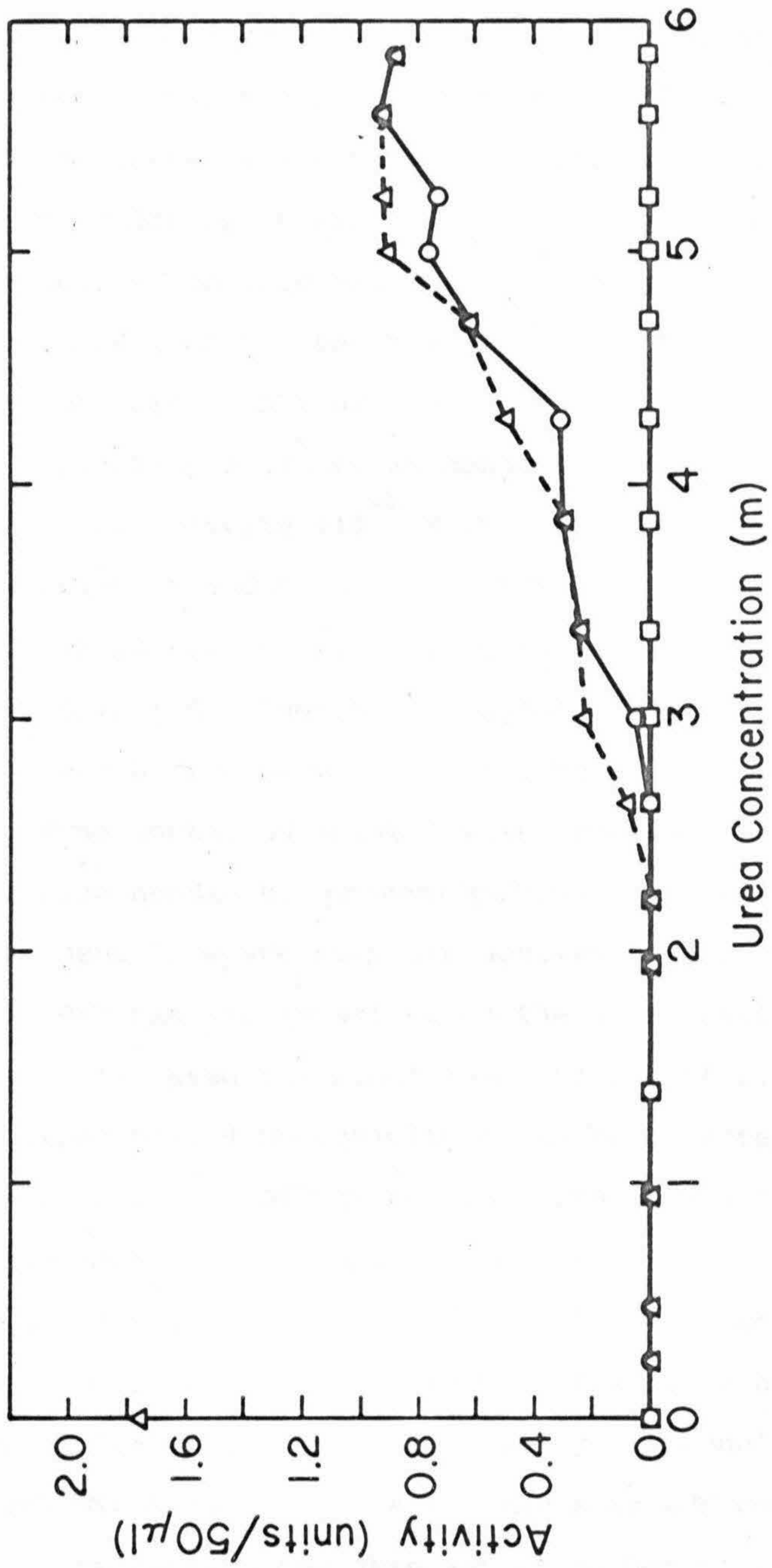
activation rate.

B. Mechanism of Activation

Several attempts, based on hypothetical activation mechanisms, were made to modify the activation process. The goal was to analyze the reactions involved in activation and to separate aggregation from activation, if possible, and produce a more soluble active enzyme.

1. Effect of denaturing agents and phenol oxidase inhibitors. Phenol oxidase was activated, at approximately 0.08 g/ml, in the presence of various amounts of urea. Enzyme assays were carried out with urea free DOPA substrate. The results (Figure 5) are shown at 2 hours when the control, containing no urea, was inactive and at 12 hours when it was fully activated. Activation was accelerated in urea concentrations of 3 M or greater while lower concentrations (0.5 to 2.3 M urea) inhibited activation up to 12 hours or longer. The highest activity reached at 5.8 M urea was 49% of the control value. The acceleration of activation at high urea concentrations,may be due to partial denaturation or dissociation of a phenol oxidase precursor. This effect is similar to the non-specific activation by denaturing agents described for several pre-phenol oxidases (see Introduction). This experiment also suggests that this effect takes place in several steps corresponding to the discontinuities in the curves of Figure 5. Perhaps two components

Figure 5. Activation of phenol oxidase in the presence of urea. Phenol oxidase, prepared by Procedure II, was activated at 0.08 g/ml in 0.02 M phosphate buffer, pH 6.7, containing various amounts of urea. The control values are shown at zero urea concentration. Activity of the samples was measured after 1 min. (squares), 2 hours (circles), and 12 hours (triangles) of activation.



Activity (units/50μl)
0.0
0.4
0.8
1.2
1.6
2.0
0
1
2
3
4
5
6
Urea Concentration (m)

of the activating system are affected by urea, one being more sensitive than the other (e.g. A_1 and A_2).

Delayed activation also takes place in the presence of the non-ionic detergent Triton X-100 (5% v/v). Sodium deoxycholate, an anionic detergent, prevents activation at concentrations from 0.5 to 5% (w/v).

Dithiothreitol (DTT or Cleland's reagent) was added to several activating extracts at concentrations which inhibit phenol oxidase activity (10^{-4} M or greater). The effects of DTT on activation under various conditions is shown in Table 1. At pH 6.65 to 6.8, DTT partially inhibits phenol oxidase activation, though the magnitude of this effect may depend on the buffer used. In experiment 3 the activation solution also contained about 5 times the ascorbic acid concentration needed to prevent quinone accumulation. For experiment 1, where complete activation curves were obtained, DTT had little effect on the time course of activation but decreased the final level of enzyme activity.

In experiment 4 no activity could be detected after activation in 10^{-4} M DTT at pH 7.8. The effect of DTT could not be reversed after 24 hours by gel filtration of the activating extract. DTT at 10^{-5} M, which does not inhibit activity, had no effect on activation (Expt. 5) at this pH. Thus, at pH 7.8 activation and activity of phenol oxidase may be inhibited at approximately the same DTT concentration. These results confirm the observation of Horowitz and Fling

Table 1.

The Effect of Dithiothreitol on Phenol Oxidase Activation.

Expt.	Activation concentration (g/ml)	buffer	DTT concentration	Hours of activation	% Control Activity
1	0.063	0.01 M NH_4COOH, pH 6.65	10^{-3} M	4	89
				24	55
2	0.65	0.1 M PO_4, pH 6.7	10^{-3} M (plus 5 x 10^{-3} M ascorbate)	20	60
3	0.65	0.02 M tris-acetate, pH 6.8	10^{-3}M	4	19
				24	13
4	0.063	0.05 M tris-HCl, pH 7.8	10^{-4} M	4	0
				24	0
5	0.063	0.05 M tris-HCl, pH 7.8	10^{-5} M	12	100%

(29) that, at pH 6 to 7, phenol oxidase activity is not necessary for the activation process. The effect of DTT at pH 7.8 may be due to irreversible inhibition of activity rather than interference with activation.

2. Effect of diisopropyl fluorophosphate (DFP) on activation. The kinetics of phenol oxidase activation, suggesting an autocatalytic process, and the partial activation of some pre-phenol oxidases by α-chymotrypsin indicated that a proteolytic step could be involved in this process. DFP, a specific inhibitor of a large group of proteases and esterases, was added to activating phenol oxidase to test the possible involvement of such enzymes.

Concentrations of DFP greater than 0.02 M are necessary to completely inhibit phenol oxidase activation at an extract concentration of 0.67 g/ml in 0.05 M tris buffer, pH 7.5. At 0.01 M, DFP has no effect on final enzyme activity but doubles the lag period compared to a control without DFP. At 0.02 M, DFP increases the lag period by 15 times and reduces the final activity to half that of the control. Inhibition in 0.1 M DFP was completely reversed by removing the DFP on a column of Sephadex G-25 (fine) or partly reversed by dilution to 0.005 M (25% activity regained). The high concentration of DFP needed to inhibit activation and the reversibility of this effect indicate that there is no serine active site protease involved in this process. However, this result does not eliminate the possible

involvement of a different type of proteolytic enzyme in activation.

C. Substrates

The oxidation of tyrosine and related compounds during activation and the subsequent quinone cross linking of proteins could greatly affect the solubility of active phenol oxidase. It is known , however, that the presence of substrates does not interfere with the activation process (29). These substrates could be small molecules or peptides not removed during enzyme preparation or released from proteins by enzymatic hydrolysis. To test these possibilities TCA soluble tyrosine was determined in extracts prepared by procedures I and II. Activation solutions contained 0.01 M NH_4COOH buffer, pH 6.65, and 10^{-3} M DTT, the latter to prevent loss of substrate molecules through melanin formation. Full activation occurred by 3.5 hours.

The results, shown in Table 2, indicate the production of tyrosine positive, TCA soluble material at a rate of 1.5 x 10^{-10} moles of tyrosine/ml/hour after the first hour of activation. This estimate is low, because tyrosine can be oxidized to DOPA and then to DOPA quinone in the presence of DTT. DTT probably inactivates the quinone by reduction or formation of a thio ester. DOPA gives approximately 1% the color yield of tyrosine in this assay. The value of 4 x 10^{-10} moles/ml/hour during the first hour of activation, when phenol oxidase activity is low, may be more nearly

Table 2.

Tyrosine content of phenol oxidase preparations.

Sample (Time after start of activation)	Tyrosine (10^{-9} moles/100µl sample)	Tyrosine (10^{-9} moles/100 µl /hour)
Procedure II Enzyme		
1 min	0.07	-------
Procedure I Enzyme		
1 min	0.28	-------
1 hr	0.32	0.04
5 hrs	0.35	0.014
23.5 hrs	0.6	0.0136
48 hrs	1.04	0.0167

accurate. In either case the production of oxidizable substrate, presumably by proteolysis, is sufficient to cause some quinone cross linking of proteins in the activation solution.

Tyrosine could also be involved in cross linking of the type found in resilin. A search for these cross linking structures, di and tri- tyrosine, in acid hydrolysates of band 2 phenol oxidase gave negative results. Chromatography on paper and cellulose phosphate was carried out as described by Andersen (18). This author also failed to find these cross links in insect cuticle either before or after sclerolization.

D. Hydroxylapatite Chromatography

A description of hydroxylapatite chromatography is included here because of its potential use in separating phenol oxidase precursors. Hydroxylapatite gel has the advantage over other adsorption media of preventing activation of phenol oxidase.

An ammonium sulfate precipitate (see preparative procedure II) equivalent to 1.25 gm of larvae was suspended directly in 30 ml of hydroxylapatite slurry in 1 mM sodium phosphate buffer, pH 6.7, and the mixture packed into a glass column. Packed bed volume was 12 ml. The gel was eluted with a continuous gradient as shown in Table 3, experiments 1, 2, and 3. There was no phenol oxidase activity in freshly eluted fractions but after an activation period

Table 3.

Elution of Phenol Oxidase from Hydroxylapatite Gel

Experiment	Elution gradient and volume	Steepness of gradient or size of steps	Phosphate concentration 1	Phosphate concentration 2	Activity recovery
1	Continuous 50 ml	0.09 molar/ml	0.060 M	1.07 M	25%
2	Continuous 50 ml	0.04 molar/ml	0.065 M	1.07 M	18%
3	Continuous 100 ml	0.03 molar/ml	0.026 M	0.85 M	7.5%
4	Step 30 ml/step	0.05 molar/step	0.028 M	0.058 M	9.6%
5	Step 30 ml/step	0.01 molar/step	0.028 M	0.03 M	0.72%

1 Phosphate concentration at the leading edge of the phenol oxidase activity peak.

2 Phosphate concentration at the maximum of the phenol oxidase peak.

of 24 hours a single, symmetrical activity peak was detected. These experiments show that the amount of potential activity recovered was a function of the steepness of the elution gradient. This is also evident in experiments 4 and 5 which used preparative procedure I and step-wise column elution. In these experiments the hydroxylapatite bed volume was 6 ml. In experiment 5,elution steps through 50 mM were pooled and concentrated on a Diaflow UM-2 membrane. This gave an enzyme activity recovery of 35% compared to 0.72% for unpooled fractions.

The behavior of inactive phenol oxidase on hydroxylapatite columns can be explained by the elution of 2 or more phenol oxidase precursor components. Phenol oxidase activity occurs only in the regions of overlap of these components.

Section II. Purification of Phenol Oxidase

A. Sucrose Gradient Centrifugation

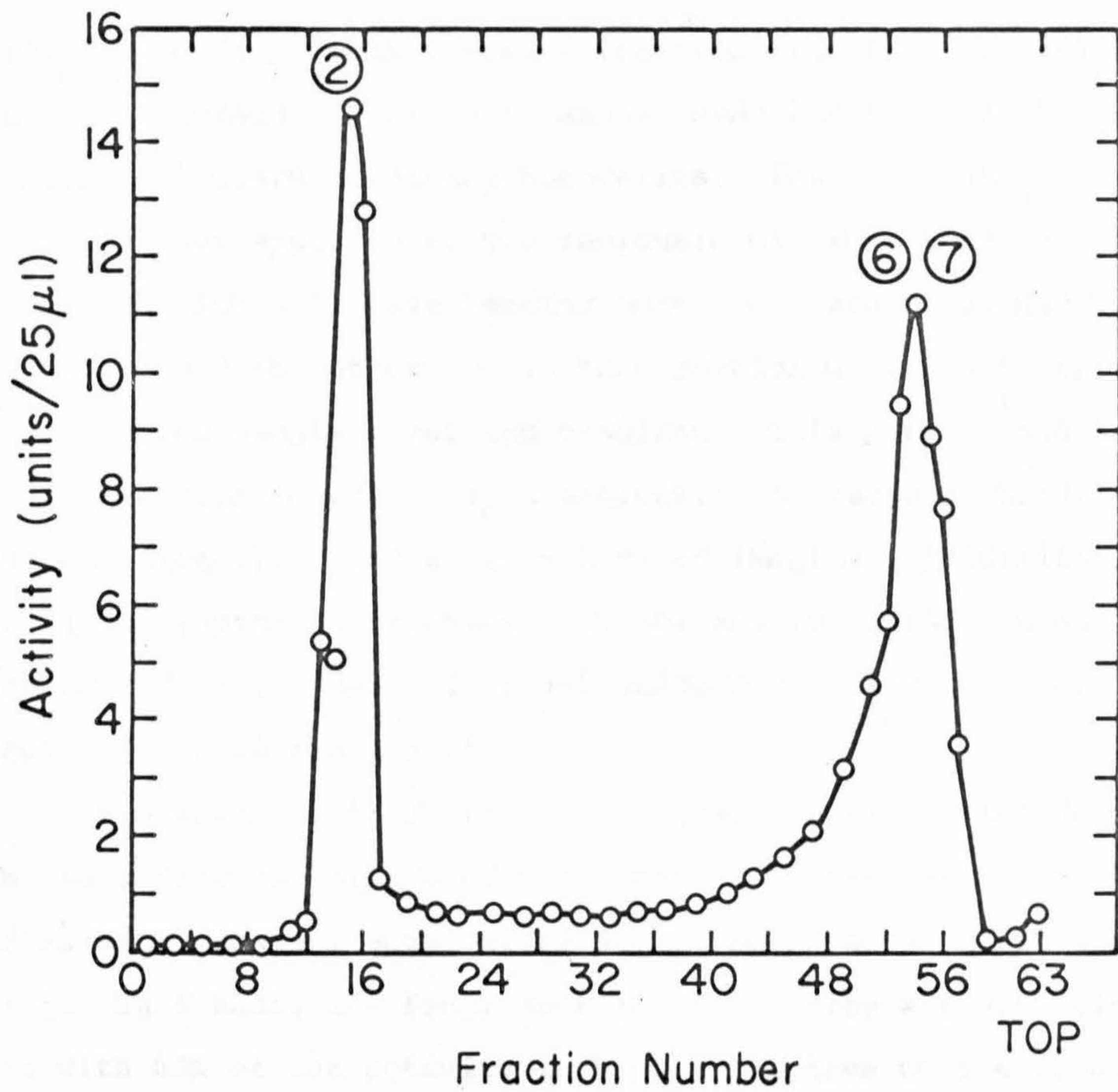
Sucrose gradient centrifugation was used to separate phenol oxidase from the bulk of the proteins in an activation extract and to analyze the products of the various activation conditions discussed in the previous section. The aim of these studies was to produce a less aggregated phenol oxidase of high purity which would be easily dissociated into its component parts. The technique of separating the large, low density phenol oxidase aggregates

on sucrose gradients was developed by Mitchell _et_ _al_. (36). A difficulty encountered with this technique was the inactivation of phenol oxidase under high centrifugal forces, probably due to shearing or compression of the aggregates. This was largely overcome by centrifugation at low speeds (e.g., 14 K rpm in the SW 25.2 rotor) and for long times (16 to 24 hours). Other methods used for purification of active phenol oxidase, including gel filtration and polyethylene glycol precipitation, were much less successful.

In this section the effects of activation concentration, pH of the activation buffer, and a reducing agent on sedimentation behavior of phenol oxidase are discussed. The main effects are changes in phenol oxidase particle size or density, or both.

1. Activation concentration. The two activation concentrations used in this work were 0.63 and 0.067 g of larvae per ml of buffer. Activation at the high extract concentration in 0.02 M phosphate buffer, pH 6.7, and centrifugation in a high density gradient (0.7 to 1.8 g of sucrose per ml of buffer, or 45 to 65% sucrose) produced the distribution shown in Figure 6. Under these conditions most of the soluble protein remains at the top of the gradient, in fractions 53 to 63. The peak of activity at the bottom of the gradient, band 2, has a density of 1.294 to 1.303 g/ml and contains 50 to 70% of the total applied enzyme activity. Approximately 10 mg of band 2 material is

Figure 6. Sucrose gradient separation of active phenol oxidase. Phenol oxidase, prepared by procedure I from <u>ebony</u> larvae, was activated at 0.67 g/ml in 0.02 M phosphate buffer, pH 6.7, for 3 hours. 5 ml samples were run on 45 to 64% gradients in the SW 25.2 rotor at 14K rpm and $4^{o}C$ for 16 hours. Fractions of 0.9 ml were collected. Sucrose solutions were made up in the activation buffer.



16
14
12
10
8
6
4
2
0
Activity (units/25μl)
②
⑥ ⑦
0
8
16
24
32
40
48
56
63
TOP
Fraction Number

produced from 10 g wet weight of _Drosophila_ larvae. The peak at the top, bands 6 and 7, was at a sucrose density of 1.132 to 1.192 g/ml. Density band numbering is as described by Mitchell _et al_. (36). Phenol oxidase bands could be easily located before fractionating the gradient by their turbid, white appearance. Band 2 was often flocculent, with sharply defined boundaries. The light band (6 and 7) sometimes appeared as two regions: one in the top of the gradient with a diffuse leading edge and sharp trailing boundary and the other a thin band remaining at the interface between the sample layer and gradient. This pattern was obtained with phenol oxidase activation extracts prepared either directly from a 56% saturated $(NH_4)_2SO_4$ precipitate or after further extraction with KCl and desalting. About 65 to 75% of the applied phenol oxidase activity could be recovered from the gradient.

The equilibrium state of this gradient was established by comparing the effect of different centrifugation times. Gradients were collected after 5, 11 and 22 hours at 24,000 x g. In 5 hours the final pattern of activity was established with 80% of the activity in band 2 relative to the 11 hour sample. The 20% increase in band 2 activity by 11 hours was due to transport of phenol oxidase activity from the top of the gradient. The gradient pattern was not significantly changed at 22 hours.

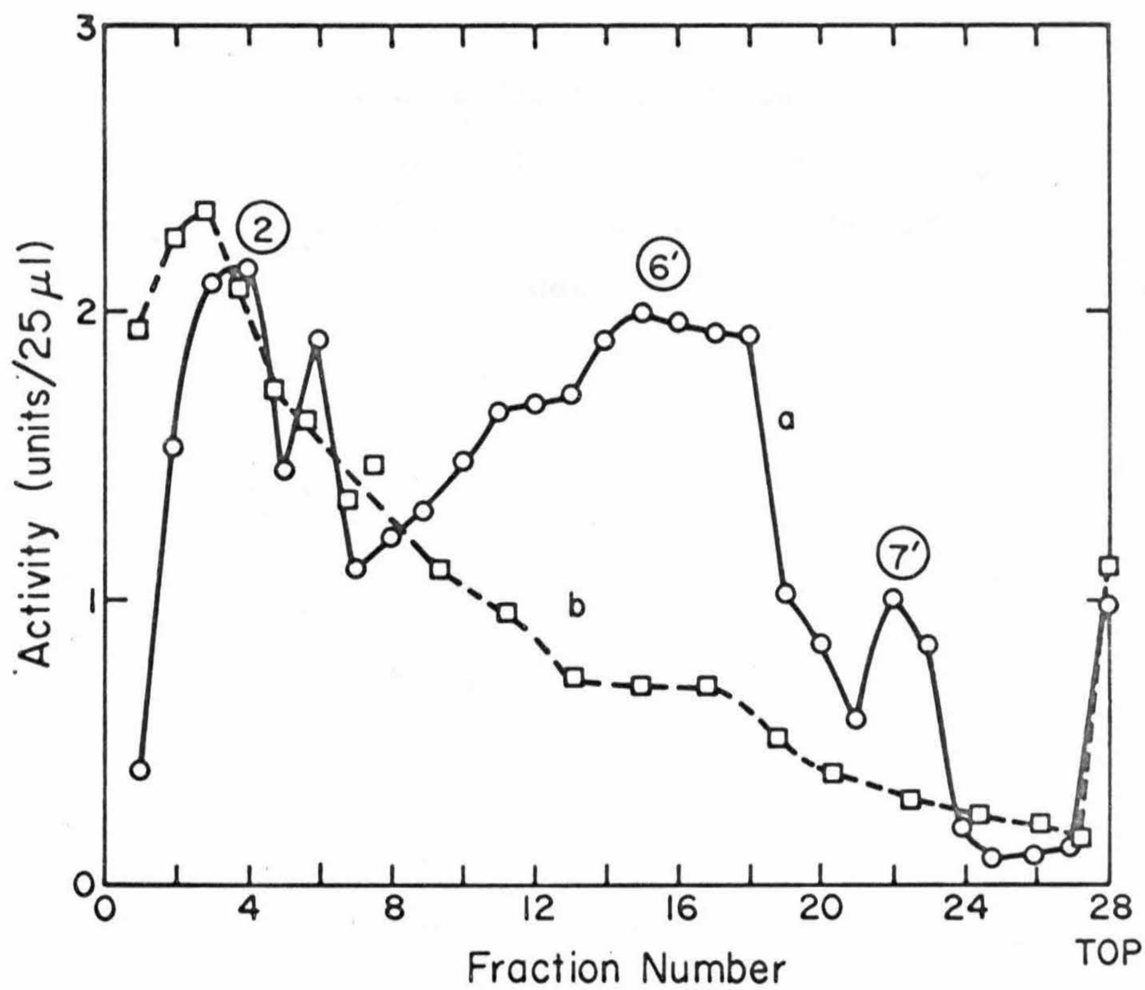
When this gradient was expanded to 5 to 64% sucrose,

the light phenol oxidase components were particularly resolved (Figure 7,a). Band 2 was present at the expected position. It was split into two peaks in this example because part of the band stuck to the side of the centrifuge tube during fraction collecting. Bands 6 and 7 appeared as a heterogeneous band through the middle of the gradient (6') and a small peak near the top (7'). Activity recovery was 100%.

When activation was carried out at 0.067 g/ml in 0.05 M tris buffer, pH 7.8,or .02 M phosphate buffer, pH 6.8,the sedimentation patterns were altered. Figures 8 and 9 show the results of these gradients, analagous to those in Figure 6 and 7, using this phenol oxidase. In Figure 8 most of the applied phenol oxidase activity (75 to 95%) remained at the top of the gradient at a median density of 1.135 g/ml. The peak of activity at the bottom of the gradient was not always present. In this experiment it is at a density of 1.285 g/ml which is significantly lighter than the density of band 2 (1.30 g/ml). When this gradient was centrifuged at 53,000 x g for an additional 6 hours the activity distribution did not change, indicating the enzyme was floating at the top of the gradient (density of 1.190 g/ml).

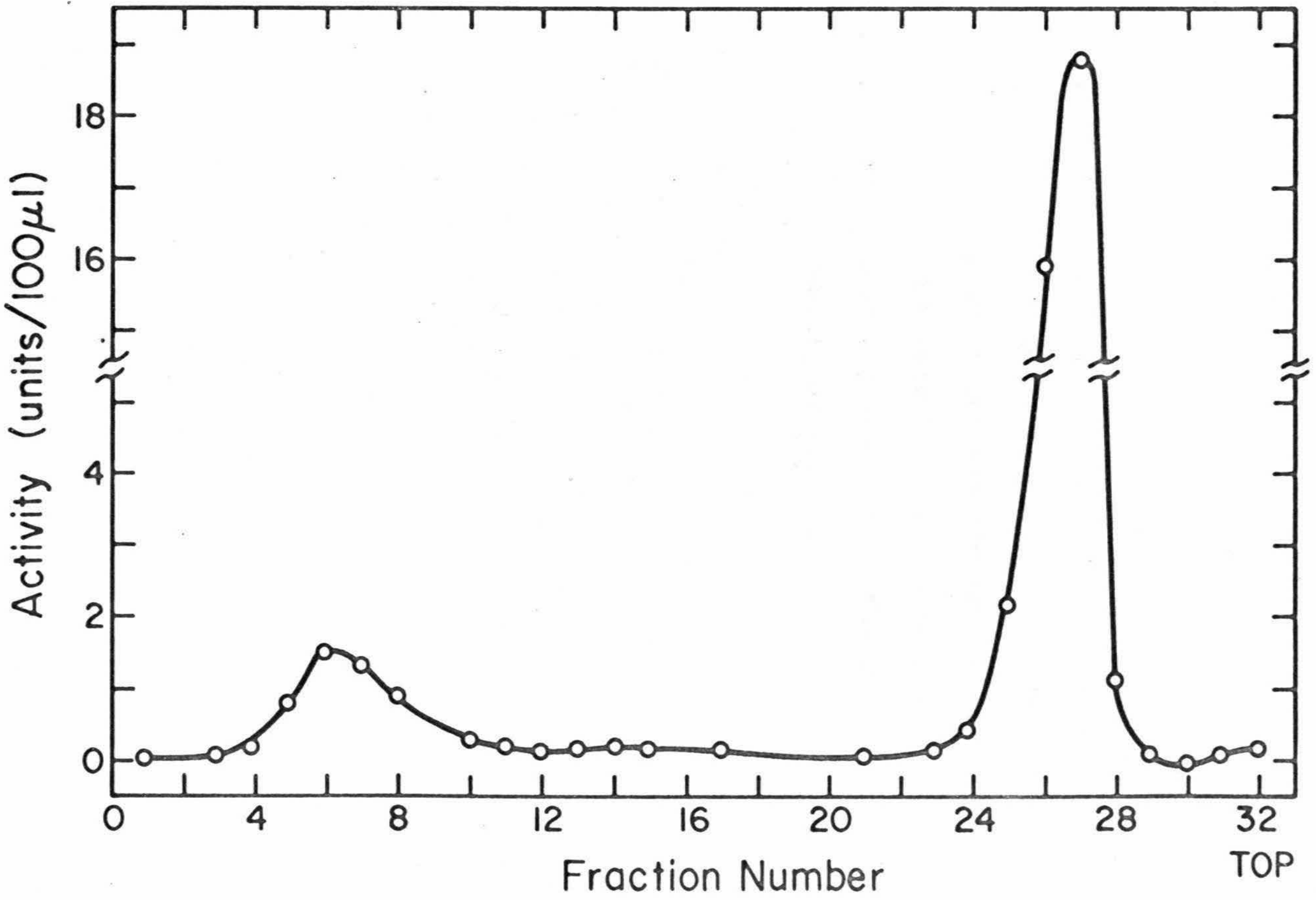
On the 5 to 64% sucrose gradient (Figure 9) the phenol oxidase activity was resolved into 3 bands (A,B,and C). The activity distribution is similar to that of Figure 7,a. The recovery of activity from this gradient was 92%. The

Figure 7. Separation of active phenol oxidase on a 5 to 64% sucrose gradient. a: The same activation extract described in Figure 5 was used in this experiment. b: Phenol oxidase, prepared by procedure II, was activated in 0.05 M tris buffer, pH 7.8, at 0.67 g/ml for 3 hours. Sucrose solutions were made up in the activation buffer. 1.85 ml fractions were collected.



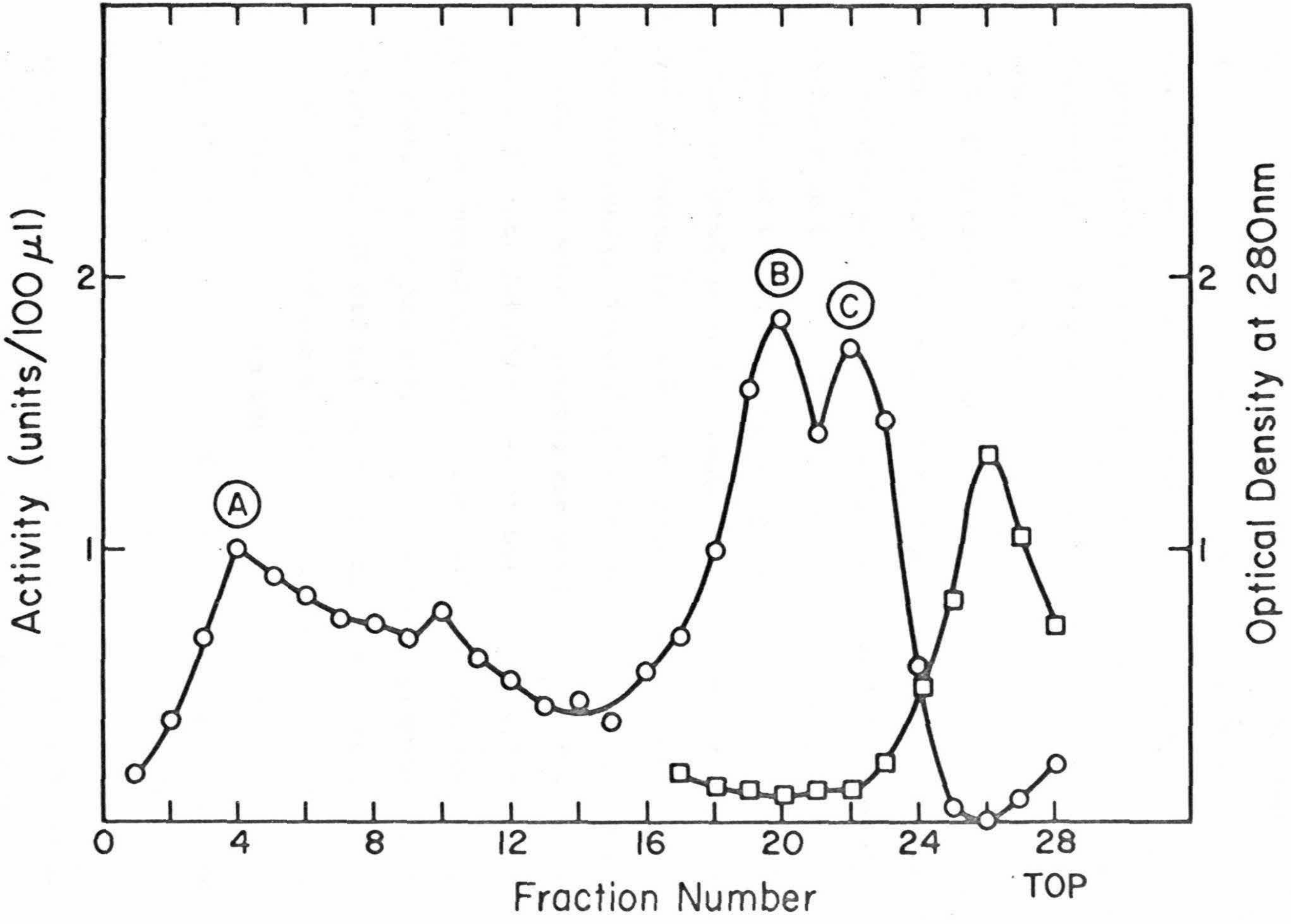
3
2
1
0
0
4
8
12
16
20
24
28
TOP
Activity (units/25 μl)
Fraction Number
2
6′
7′
a
b

Figure 8. Equilibrium distribution of phenol oxidase on a sucrose gradient. Phenol oxidase was prepared by procedure II and activated at 0.063 g/ml, pH 7.8. See text for additional details.



Activity (units/100μl)
18
16
4
2
0
0
4
8
12
16
20
24
28
32
TOP
Fraction Number

Figure 9. Separation of phenol oxidase on a 5 to 64% sucrose gradient. The preparation was the same as used in Figure 7. Circles represent activity and squares the optical density at 280 nm.

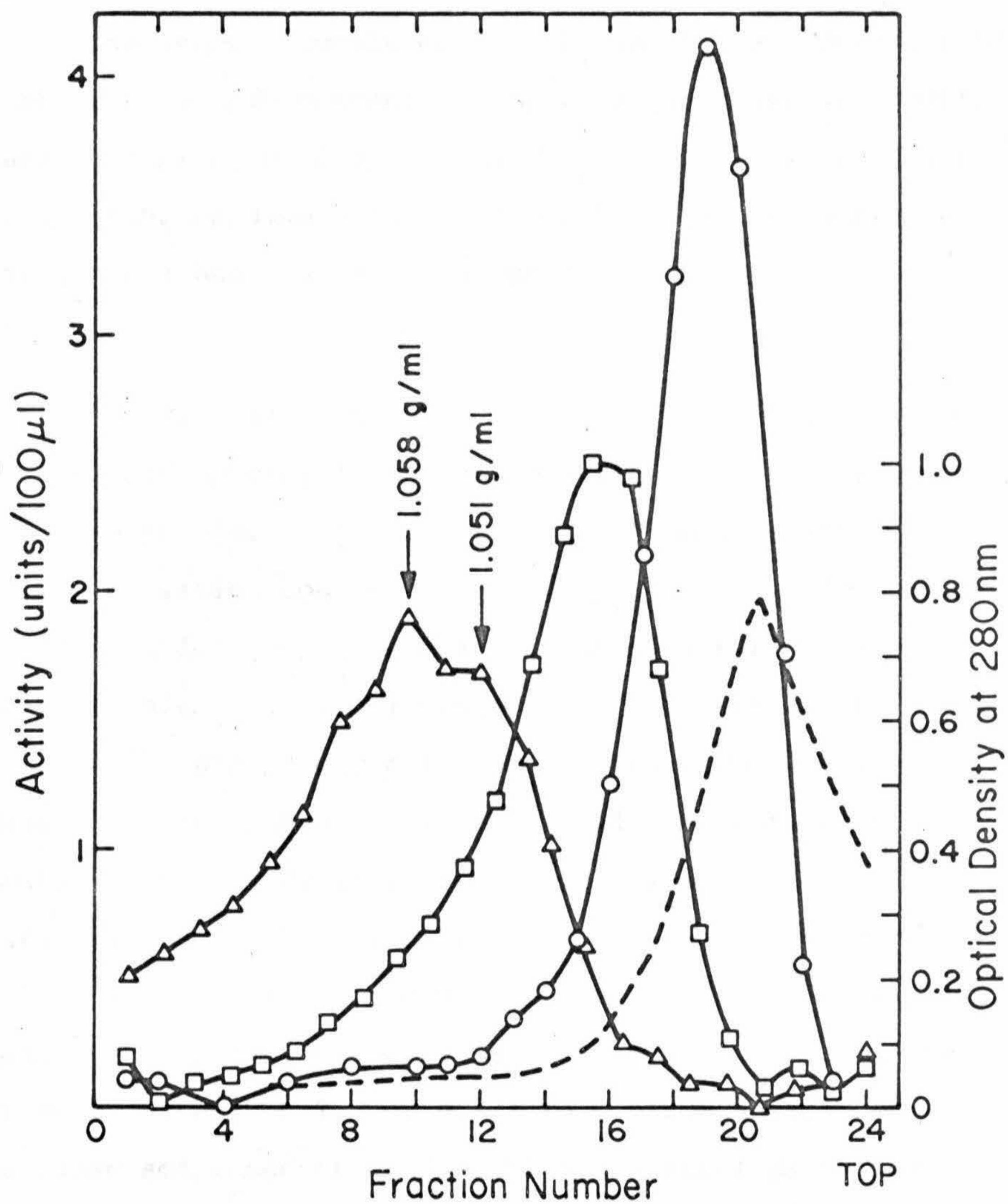


Activity (units/100 μl)
Optical Density at 280nm
Fraction Number
TOP
A
B
C
0
4
8
12
16
20
24
28
1
2

enzyme in bands B and C is less dense than 45% sucrose (1.182 g/ml), as shown in Figure 8, and either small in size or lighter than approximately 25% sucrose, (about 1.10 g/ml) which is the concentration in fractions 17-18 of the gradient in Figure 9. A comparison of Figures 8 and 9 shows a larger percentage of the total activity in band A (Fig. 9) than predicted from Figure 8. This peak of phenol oxidase activity may be an artifact produced by the transport of the enzyme into the low density region of the gradient. Centrifugation through the sucrose gradient may increase the density of the phenol oxidase aggregates.

Further studies of the phenol oxidase produced at low activation concentration were carried out on 5 to 20% sucrose gradients. Typical results are shown in Figure 10. These gradients were collected and assayed after 6,12, and 22 hours of centrifugation. About 60% of the applied activity was recovered. The phenol oxidase peak seen in these gradients is probably equivalent to components B and C in Figure 9. It did not reach an equilibrium position during the course of the experiment but appeared to be slowly sedimenting to the bottom. This was verified by adding the enzyme sample uniformly through the gradient and centrifuging at 25,000 x g for 16 or 24 hours. Again all of the phenol oxidase activity sedimented to the bottom of the tube with a 12% loss in 16 hours and 23% by 24 hours due to pelleting on the bottom of the tube. However, this

Figure 10. Time course of sedimentation of phenol oxidase on a 5 to 20% sucrose gradient. Preparation was as in Figure 7. The gradients were centrifuged at 14K rpm in the SW 25.2 rotor. 1.85 ml fractions were collected. Circles represent the enzyme activity after 6 hours; squares, 12 hours; and triangles, 22 hours of centrifugation. The broken line represents the optical density at 280 nm after 22 hours of centrifugation. Sucrose densities of two fractions are shown for the 22 hour sample.



Activity (units/100μl)
Optical Density at 280nm
Fraction Number
TOP
1.058 g/ml
1.051 g/ml
0
4
8
12
16
20
24
1
2
3
4
0.2
0.4
0.6
0.8
1.0

phenol oxidase seemed to reach a density equilibrium position, on a 10 to 45% sucrose gradient, of 1.08 to 1.09 g/ml.

The general result of activation at low concentration, relative to high concentration, is a reduction in density and perhaps size of up to 50% of the phenol oxidase. All of the phenol oxidase produced seems to have the characteristics of bands 6 and 7 with an insignificant amount of band 2 enzyme.

2. Activation pH. The effect of the pH of an activation extract on the sedimentation behavior of phenol oxidase is also a function of activation concentration. At high activation concentration the pH greatly affects the sedimentation of phenol oxidase, as shown in Figure 7, a and b. The higher pH (7.8) caused the disappearance of peaks 6' and 7' and produced a high density form of the enzyme which was deposited on the bottom of the centrifuge tube. Activity recovered in this example (b) was 44% from the gradient and another 11% from the bottom of the tube.

At low activation concentration the sedimentation pattern of phenol oxidase, as seen in Figure 8, was the same at either pH 7.8 or 6.7. To confirm this, phenol oxidase activated at 0.063 g/ml and several pH values (6.7, 7.3, 7.8 and 8.3) was sedimented on 5 to 20% sucrose gradients. All activity profiles were the same as shown in Figure 10 (22 hours) with recoveries of 50 to 70% of the

applied activity. Activation concentration rather than pH seems to be the important factor in producing this low density enzyme which is also in a reduced state of aggregation (see Section III, A of Results).

3. The effect of dithiothreitol. If phenol oxidase activity were involved in aggregation, inhibition of this activity should affect sedimentation behavior of the enzyme on sucrose gradients. However, activation in the presence of 10^{-5}M DTT at pH 7.8 or 10^{-3} DTT at pH 6.7 did not qualitatively affect the banding patterns on sucrose gradients. This suggests that under these condition phenol oxidase activity is not involved in the aggregation process. It also indicates that disulfide bonds formation is not responsible for aggregation.

B. Purity of Phenol Oxidases from Sucrose Gradients

It is difficult to determine absolute purity of active phenol oxidase because of its size heterogeneity and variations in density. Several indirect determinations were made to measure relative purity and contamination by non-phenol oxidase proteins. Table 4 shows the specific activities of several phenol oxidase preparations. These are based on maximum activities measured in a given sample. Ten grams of larvae yield 1500 mg of soluble protein which is purified to about 220 mg (7 fold) in the activation extract. Gradient centrifugation then provides up to 8 fold

Table 4.

Specific Activities of Phenol Oxidases

Sample	Preparation procedure	Activation concentration (g/ml)	Sucrose Gradient	Fractions analyzed	Specific activity (U/mg protein)
1	II	0.62	none	active enzyme	88
2	I	0.67	none	active enzyme	99,81*
3	II	0.62	0.7 to 1.8 g sucrose/ ml buffer	band 2	442
4	I	0.67		band 2	512,527*
5	II	0.063	5 to 20% sucrose	major peak at 16 hr. See Fig. 10	654
6	II	0.063		A, fractions 2-12	470
7	II	0.063	See Fig.9	B, fractions 17-20	697
8	II	0.063		C, fraction 22	325
9	I	0.67	See Fig.7, a.	6′, fractions 13-16	150

* Duplicate values were obtained from different experiments.

purification of the phenol oxidase. Specific activities of band 2 enzyme are probably not significantly different (samples 3 and 4) but the phenol oxidase in sample 5, which was activated in a dilute extract, has about 35% greater specific activity. The phenol oxidase in samples 7 and 8 represent subcomponents of sample 5 but differ greatly in specific activity. The highest specific activities observed (5 and 7) both represent slowly sedimenting enzyme from dilute activation conditions. The lowest specific activity (sample 9) is of the heterogeneous band from a concentrated activation extract.

These differences in specific activity could be due to differences in purity or artifacts of centrifugation in which activity is destroyed or inhibited due to aggregation of the phenol oxidase. Another possible explanation is that the phenol oxidases formed under these various conditions are of different subunit compositions, thus have different specific activities.

An experiment was performed to determine directly the contamination of band 2 phenol oxidase by non-phenol oxidase proteins. This possibility was suggested by the flocculent, sticky nature of the enzyme. _E_. _coli_ protein labelled with ^{14}C-leucine was added to a _Drosophila_ extract during the 1.5 M KCl extraction (see Procedure I, section 2, Materials and Methods). The enzyme was subsequently activated at a concentration of approximately 0.7 g/ml in 0.02 M

phosphate buffer, pH 6.7, and centrifuged on a 45 to 64% sucrose equilibrium gradient. The ^{14}C labelled protein was about 0.03% of the total protein in the activation extract and the specific activity of the mixture was 12.3 cp./ug of protein.

Band 2 phenol oxidase from the gradient contained 5% of the ^{14}C-leucine protein and 4% of the total protein applied in the activation extract. If the ^{14}C labelled protein is considered equivalent to all non-phenol oxidase proteins in the activation solution, the maximum heavy band contamination would be very high. An aliquot of heavy band enzyme was washed in distilled water until the supernatant radioactivity was at background level. About 85% of the counts were removed by this washing procedure, leaving about 15% by weight contamination of the phenol oxidase. These results show that phenol oxidase is probably contaminated with a small amount of non-phenol oxidase protein. It is also evident that E. coli protein may not be an adequate substitute for Drosophila proteins.

Section III. Properties of Phenol Oxidase.

A. Aggregation.

As described earlier, aggregation is a constant property of active phenol oxidase, though the degree of aggregation may vary with activation conditions. High

extract concentrations during activation produce a large heterogeneous enzyme which can be rapidly sedimented at 25,000 x g. This enzyme was too large to enter chromatographic gel beds or 5% acrylamide electrophoresis gels and only 10 to 20% of the activity passed through a Millipore filter (0.45 micron pore size). The degree of aggregation increased with time so that the enzyme eventually precipitated out of solution. In contrast, activation at pH 7.8 in dilute extracts produced smaller aggregates, most of which could be recovered from gel filtration columns, though still large in size (see below). About 75% of this phenol oxidase activity passed through a Millipore filter.

Several experiments were conducted to reduce the aggregation state of fully activated phenol oxidase. 5M urea, when added to phenol oxidase activated at 0.6 to 0.8 g/ml in 0.02 M phosphate buffer, pH 6.7, reduced enzyme activity by about 46%. To determine the state of the remaining activity the extract was centrifuged on a 5 to 20% sucrose gradient, 5M in urea, at 7,200 x g for 1 hour. Under these conditions most of the activity in a control sample had sedimented to the bottom of the gradient tube. However, all activity remaining in 5 M urea was at the top of the gradient. The effect of urea, then, was to either reduce the density of the phenol oxidase activity to less than 1.10 g/ml or to reduce its size.

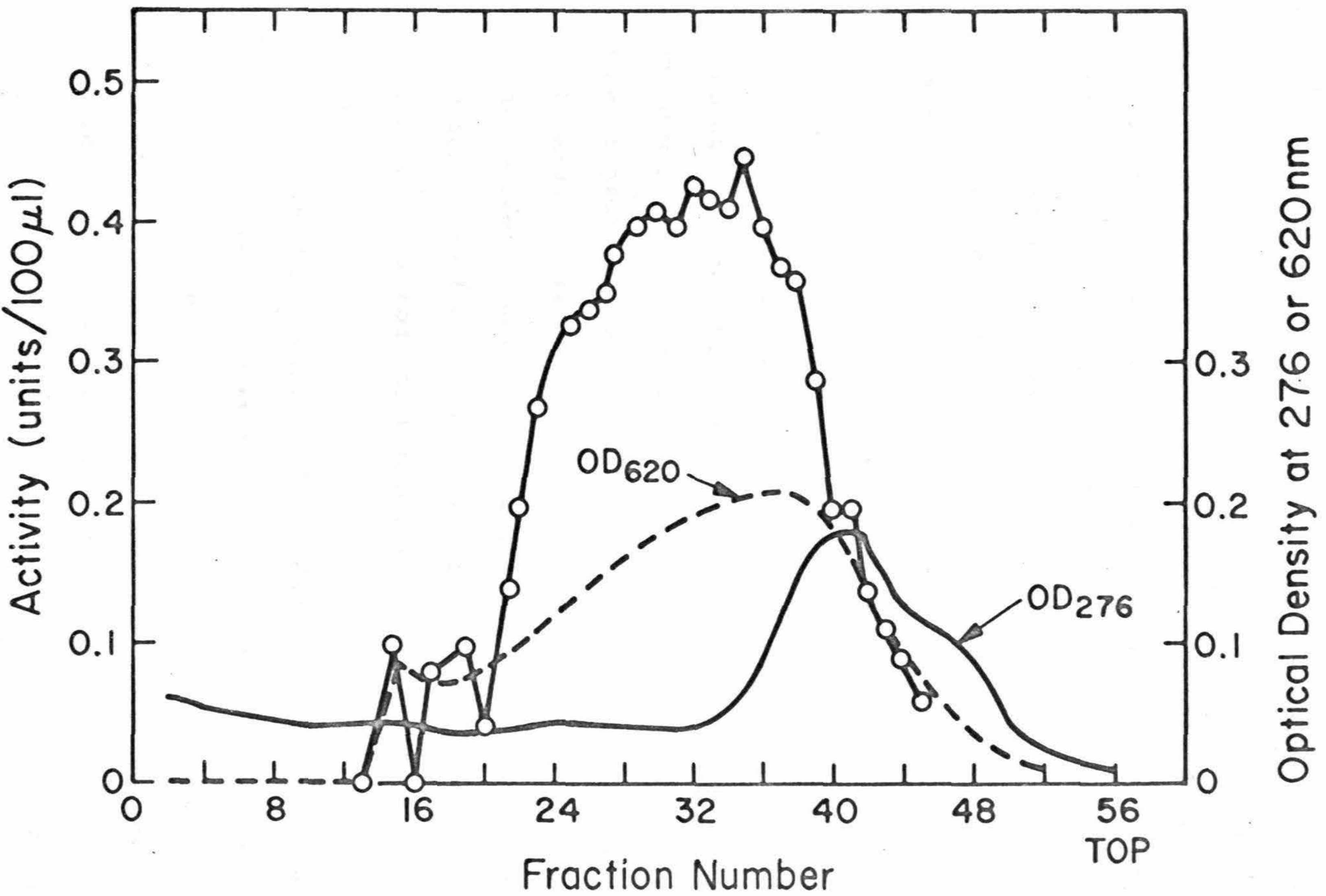
Phenol oxidase activated from a dilute extract

(0.067 g/ml) and pH 7.8 was incubated in 5% (w/v) NaDOC at $5^{o}C$. Sodium deoxycholate has no inhibitory effect on phenol oxidase activity and seems to increase it slightly. Samples were centrifuged at 100,000 x g for 30 min, after various incubation times. In a typical experiment the supernatant contained 59% of the initial activity after 24 hours of incubation and 78% after 72 hours. The controls, lacking NaDOC had no supernatant enzyme activity. Sodium deoxycholate at 0.5% (w/v) gave a similar result indicating that 60 to 70% of the phenol oxidase was either low in density (less than 1.012 g/ml) or reduced in size.

To determine the density of this enzyme a NaDOC treated sample was layered on a 5 to 40% sucrose gradient, without the detergent, and centrifuged at 100,000 x g (max. force) at $5^{o}C$. After 13 hours about 30% of the applied activity had reached density equilibrium position of about 1.125 to 1.145 g/ml, while the rest had sedimented to the bottom of the tube. The density of the active phenol oxidase was then greater than 1.012 g/ml indicating that NaDOC treatment must have reduced enzyme aggregation.

The size of NaDOC treated phenol oxidase was also analyzed by chromatography on an agarose gel filtration column. The result, shown in Figure 11, for a Sepharose 2B column in 0.5% NaDOC, show a large, heterogeneous included peak. The exclusion limit of this gel is 25×10^{6} m w. using polysaccharide standards (see: Technical Data Sheet

Figure 11. Chromatography of sodium deoxycholate treated phenol oxidase on Sepharose 2B. The sample of activated phenol oxidase was treated with 0.5% NaDOC in 0.05 M tris buffer, pH 7.8, for more than 24 hours and filtered through a 0.45 micron Millipore filter which removed 20% of the activity. 1.75 ml was applied to a 0.35 x 1.9 cm bed of Sepharose 2B in 0.005 M Tris buffer, pH 7.8, and 0.5% NaDOC at $5^{o}C$. The column was run under a 24 cm pressure head and with a flow rate of 6.5 ml/hr. 2.2 ml fractions were collected. The circles represent enzyme active; the solid line, total protein (OD_{276}); and the broken line, Blue dextran (OD_{620}).


Activity (units/100μl)
0.5
0.4
0.3
0.2
0.1
0
Optical Density at 276 or 620nm
0.3
0.2
0.1
0
OD620
OD276
0
8
16
24
32
40
48
56
TOP
Fraction Number

No. 12, Pharmacia Fine Chem., Inc.) and probably greater using globular proteins. A difficulty in interpreting these results is the lack of information on the effect of NaDOC on the gel filtration properties of agarose. Thyroglobulin was used for a standard in this experiment and gave a K_{av} of 0.58 (fraction 32). This agrees closely with the K_{av} (0.55) predicted from the Pharmacia specifications for a molecular weight of 670,000 (see: Sepharose, agarose gels in bead form, Fig.3, Pharmacia Fine Chemicals, Inc.). Using this published standard curve, the elution profile can be calibrated at about 2×10^6 m w for fraction 22 and about 10^5 m w for fraction 40. Phenol oxidase activity, then, would have molecular weights between 0.5 and 1.5×10^6. The major protein peak (OD_{276}) is clearly smaller than the phenol oxidase (less than 5×10^5 m w). The behavior of the bulk proteins on other gel filtration columns without detergents indicates a mean molecular weight greater than 1 to 2×10^5 for most of the protein in the activation extract.

To extend these observations, NaDOC and the non-ionic detergent Trition X-100 were added to the highly aggregated band 2 phenol oxidase. Band 2 enzyme was diluted five times with 0.05 M tris buffer, pH 7.8,to raise the pH and to dilute out the sucrose. Detergents were added and after 6 hours and 26 hours of incubation at 25°C, the solutions were assayed for activity, centrifuged at 100,000 x g

(max. force) for 1 hr, and the supernatants assayed. The results are shown in Table 5. Sodium deoxycholate at 5% was most effective in solubilizing the enzyme activity (48%). In some cases the supernatant activity was as high as 65% of the total. Note the enhancement of activity by the detergents. The amount of phenol oxidase in the supernatant was not a function of solution density in these experiments. This was tested by adding 10% sucrose to duplicate samples before centrifugation.

These results show that up to 65% of the heavily precipitated band 2 phenol oxidase can be solubilized by detergent treatment. However, aggregation seems to continue in the presence of detergent. If the 100,000 x g supernatant is stored at 4°C for a day, 20% of the activity is lost by centrifugation at 100,000 x g. This is not due to inactivation during centrifugation, for the lost activity can be recovered by resuspending the pellet. Sodium deoxycholate protects the enzyme activity from centrifugation loss, since without the detergent only 10 to 15% of the pelleted activity can be recovered under these conditions. Freezing the supernatant at -10°C for several days also results in a 50% loss of activity upon recentrifugation.

B. Dissociation.

Initial attempts to dissociate phenol oxidase were carried out with band 2 enzyme from equilibrium density

Table 5.

Solubilization of Phenol Oxidase Activity by Detergents.

(a) after 6 hours at 25°C

Detergent	Total activity* units/10µl	Supernatant activity* units/10µl	% Solubilized activity
none	1.84	0.28	15
5% T X-100	1.9	0.12	6
10% T X-100	1.83	0.10	5
15% T X-100	1.57	0.08	5
5% NaDOC	-----		
7.5% NaDOC	2.06	0.69	33
10% NaDOC	-----		
15% NaDOC	1.90	0.60	32

(b) after 26 hours at 25°C

Detergent	Total activity* units/10µl	Supernatant activity* units/10µl	% Solubilized activity
none	1.43	0.22	15
5% T X-100	1.19	0.36	30
10% T X-100	1.58	0.58	37
15% T X-100	1.57	0.53	34
5% NaDOC	1.71	0.82	48
7.5% NaDOC	1.70	0.67	39
10% NaDOC	1.62	0.65	40
15% NaDOC	1.55	0.56	28

* Centrifugation was at 100,000 x g for 1 hour. Total activity is for the sample before centrifugation.

gradients. This was washed and lyophilized, then treated under denaturing conditions for 24 hours at room temperature. The protein content of the 100,000 x g (max.force) supernatant was analyzed and compared to the dry weight of the extracted phenol oxidase. One milligram of the dry enzyme gave a Folin-Lowry reaction equivalent to 0.98 $\pm$ 0.033 (S.E.M.) mg of BSA. The results of these experiments are presented in Table 6, column A. These results suggest that a large percentage of this enzyme was covalently cross-linked and not susceptable to dissociation. Later, these extraction experiments were repeated with freshly prepared band 2 phenol oxidase. Table 6, column B shows that this material contained much more extractable protein. The lower extractability of lyophilized phenol oxidase was probably an artifact due to secondary cross linking of the enzyme molecules during drying. These results indicate that there may still be an insoluble, cross linked matrix of protein (up to 25%) in non-lyophilized band 2 phenol oxidase.

To determine the qualitative nature of this dissociated phenol oxidase,samples were analyzed by SDS acrylamide gel electrophoresis. Several preparative techniques were tried for the protein samples and the most successful was to concentrate phenol oxidase from a sucrose gradient on an Amincon UM-30 membrane, add SDS and β-mercaptoethanol, each to 0.1%, and heat for 20 min at 100°C. Steps involving dialysis of the sample in cellophane tubing were avoided

Table 6.

Solubilization[‡] of Phenol Oxidase
by Denaturing Agents.

Reagent	Percent of protein solubilized from band 2 enzyme	
	A Lyophilized	B Freshly Prepared
98% formic acid	37	75
9 M urea, 5% SDS, 5% β-mercaptoethanol	34.5 (23)*	59
6 M urea	7.9 (18.5)*	----
2 M NaSCN	7.2	----

[‡]Soluble protein remained in the supernatant after 30 min at 100,000 x g (max. force), at 24°C.

*After 1 hour at 100°C.

because of up to 50% loss of protein. The amount of protein applied to an electrophoresis gel was not measured directly but calculated from enzyme activity before adding SDS. The specific activities used were obtained from the data in Table 4. except for gel number 5 which was calculated from an activity of 550 units/mg of protein. Since the degree of dissociation of the phenol oxidase seemed to vary with the source of the enzyme ,the amount of protein in the applied samples was always greater than that which entered the gel. Some samples did not give any protein bands in these experiments. The results are summarized in Table 7 and Figure 12. Figure 13, a spectrophotometer tracing of one of the stained SDS gels, illustrates the complexity of the pattern of dissociated phenol oxidases. There are many closely spaced bands differing by about 2,000 in molecular weight. A major set of bands is the triplet seen between 79,000 and 85,000 m.w. There is also a number of bands of molecular weight greater than 130,000. The results show that some sources of phenol oxidase are dissociable into many species or aggregates. These patterns may not, however, represent molecular weight distributions but may reflect the binding of SDS to non-protein parts of the phenol oxidase complex or fragments of these. The binding of SDS to protein has been shown to be proportional to molecular weight (57) but its interaction with lipoproteins has not been reported. Highly purified A_1, which

Table 7.

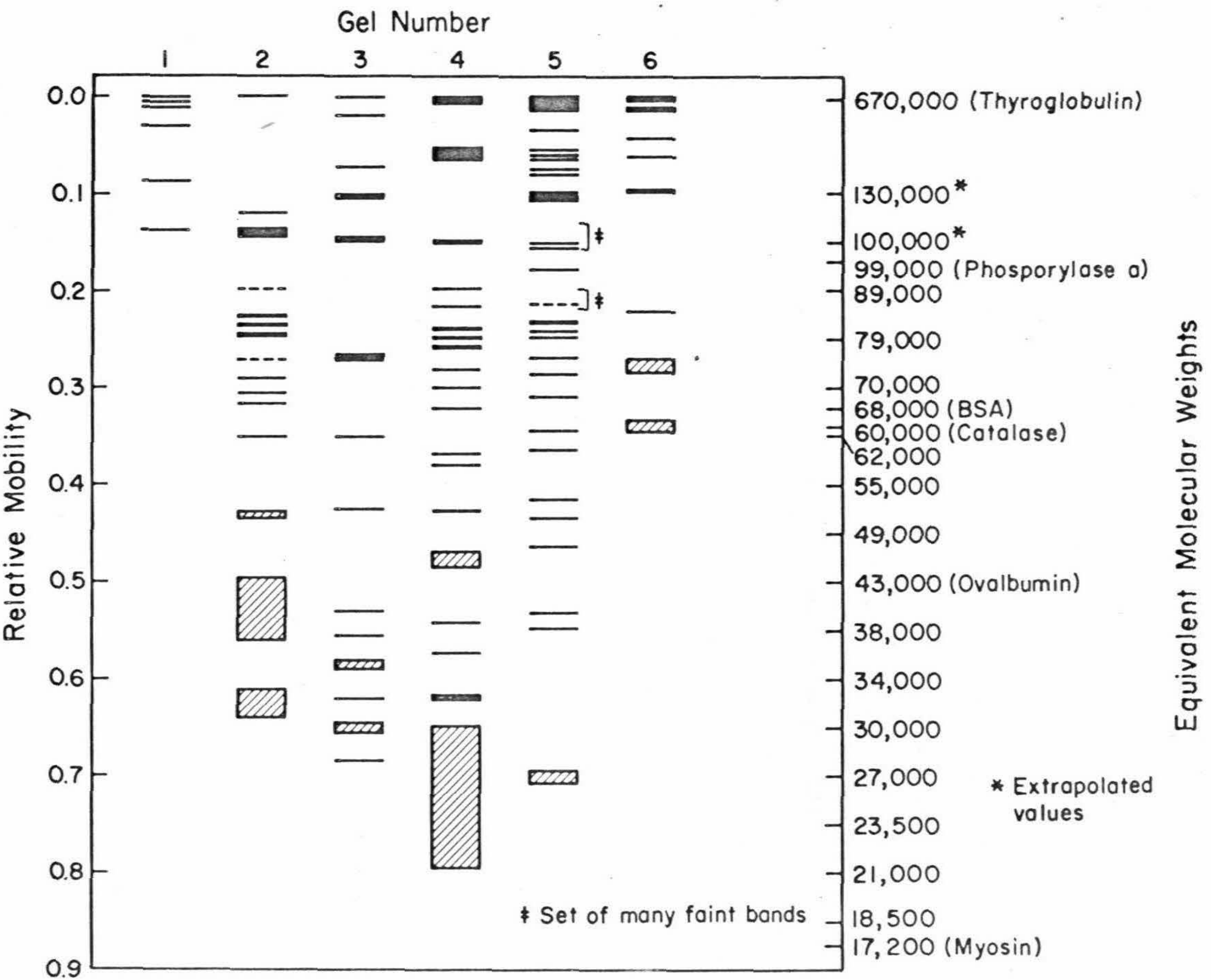
Samples used for SDS Acrylamide Gels

as Shown in Figures 12 and 13.

Gel no.	Source of Phenol Oxidase	Sample size (µg of protein)	Comments
1	As in Figure 10	24 µg	
2	As in Figure 10	36 µg	
3	Band A, Figure 8	28 µg	
4	Band 6' Figure 7	52 µg	Fractions 11-18
5	Band 6' Figure 7	52 µg	
6	Low density phenol oxidase, 5 - 64% sucrose gradient.	18 µg	Activated at 0.063 g/ml in 0.05 M tris, pH 7.8, approx. 10^{-7} M DTT.
Not shown	Peak B, Figure 9	18 µg	One protein band at origin
Not shown	Peak C, Figure 9	20 µg	One protein band at origin
Not shown	Band 2, Figure 6	51 µg	One protein band at origin

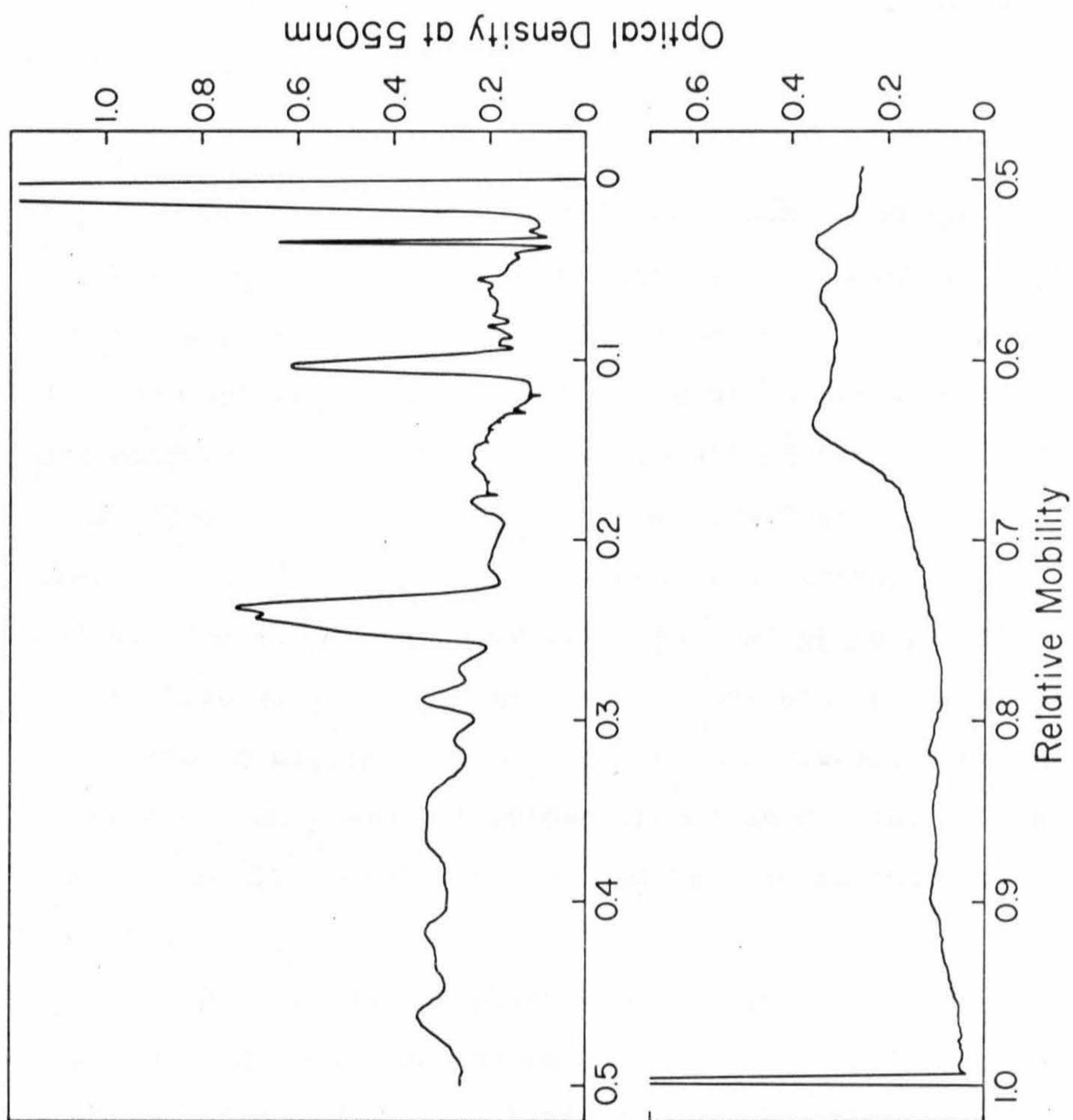
Figure 12. Protein bands on SDS acrylamide gels of phenol oxidase. These patterns are drawn from measurements made of stained SDS gels. Migration distances have been converted to relative mobilities. On the right is a calibration curve of equivalent molecular weights determined from standard proteins. Note that catalase (60,000 m w.) deviates slightly from the standard curve. Details on the samples used are presented in Table 7.

Cross hatched or dotted bands indicate very light protein staining.



Gel Number
1
2
3
4
5
6
Relative Mobility
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Equivalent Molecular Weights
670,000 (Thyroglobulin)
130,000*
100,000*
99,000 (Phosporylase a)
89,000
79,000
70,000
68,000 (BSA)
60,000 (Catalase)
62,000
55,000
49,000
43,000 (Ovalbumin)
38,000
34,000
30,000
27,000
23,500
21,000
18,500
17,200 (Myosin)
* Extrapolated values
‡ Set of many faint bands
‡
‡

Figure 13. A densitometer recording of the SDS acrylamide gel No. 5 also shown in Figure 12. Mobilities are relative to that of brom phenol blue.


Optical Density at 550nm
Relative Mobility
0
0.2
0.4
0.6
0.8
1.0
0.1
0.3
0.5
0.7
0.9

was shown to be homogeneous by several criteria, gives 2 closely spaced bands at 77,000 ± 2,000 m w on SDS acrylamide gels (58). Occasionally a band also appears at about 150,000 m w.

C. Chemical Composition

1. Amino acid composition. Washed and lyophilized band 2 (ebony) and band 3 (wild type) phenol oxidases were hydrolyzed in 6 N HCl for amino acid analysis. The results are presented in Table 8. Weight yields of amino acids were computed, based on the total weight of the enzyme sample. These values are low because of excessive destruction of aromatic amino acids in the presence of carbohydrates or lipids. The compositions of wild type and ebony phenol oxidases are very similar and do not show unusual proportions. There were no significant amounts of amino sugars detected though there were several unidentified minor peaks. Minimum enzyme molecular weights calculated from these data are 15 to 20,000.

2. Lipids and phosphate. Evidence for lipid components of band 2 phenol oxidase comes from the detection of phosphate and fatty acids. The phosphate content is 8.4×10^{-8} moles per mg of dry phenol oxidase or about 8 molecules per 100,000 m.w. Partial methanolysis of a phenol oxidase sample and gas chromatography of the extracted methyl esters

Table 8.

Amino Acid Composition of Phenol Oxidase

Amino Acid	Content (mole %)	
	band 2	band 3
asp	9.6	9.8
thr	7.4	7.5
ser	5.4	5.4
glu	11.9	11.8
pro	5.9	5.9
gly	12.2	11.2
ala	7.5	7.5
val	3.6	4.7
cys	1.9	1.6
met	1.4	0.9
ilu	4.7	5.0
leu	7.2	7.4
tyr	2.8	2.9
phe	4.5	4.4
lys	7.6	7.6
his	1.7	1.4
arg	4.8	4.8
Yield (wt.%)	66.5%	60.1%

shows a multiplicity of fatty acids in the enzyme. Several of these peaks, shown in Figure 14, have been tentatively identified by correspondance with standard compounds. The results are qualititatively compatible with those of Kieth (57) for the fatty acid composition of whole *Drosophila* adults.

3. Sugars. Anthrone reactions were carried out with lyophilized band 2 phenol oxidase to determine the amount of hexose sugars present. The value obtained was about 3% by weight. A control using 10 mg of BSA gave a negligible anthrone color reaction.

D. Substrate Specificity.

All of the preceeding results have been given in terms DOPA oxidase activity. In some experiments tyrosine was also used as substrate to characterize the phenol oxidase peaks from sucrose density gradients. The results are shown in Table 9. Samples 1 and 2 are very similar in their relative abilities to oxidize tyrosine and DOPA. Samples 4 through 8 are sucrose gradient fractions corresponding to Sample 2, a dilute activation extract, and 9 through 11 to Sample 1, a concentrated activation extract. Samples 4 and 5 show that the composite band 6 and 7 contains components with at least a ten fold difference in their tyrosinase activities. This lack of tyrosinase activity in band 7 has also been observed by Mitchell *et al*. (36). When this

Figure 14. Analysis of phenol oxidase fatty acid composition by gas-liquid phase chromatography. Tentative assignments of several peaks were made by comparison with standard methyl esters of fatty acids. These are shown in parentheses as the number of carbon atoms in the standard fatty acids.

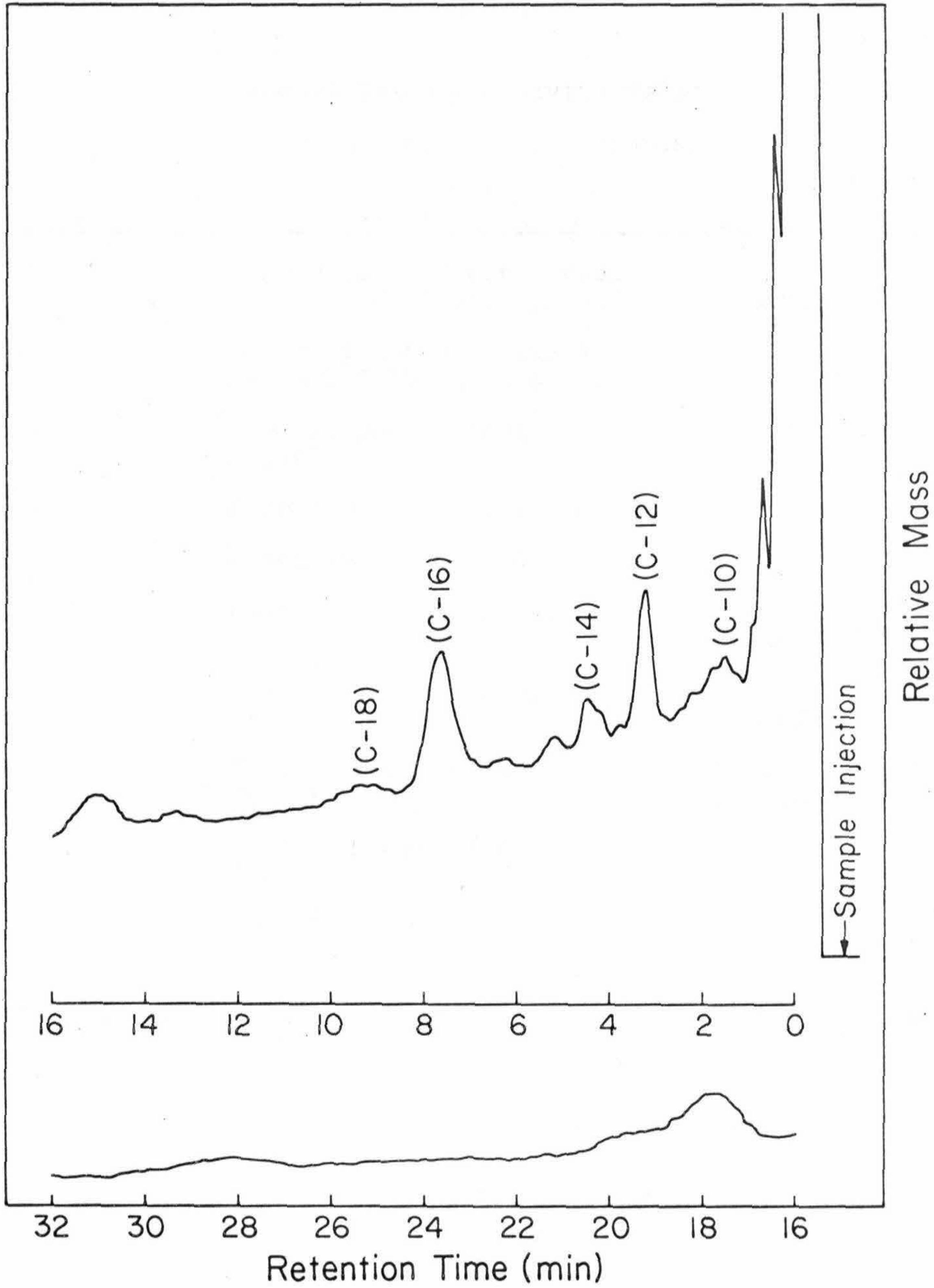

Relative Mass
Sample Injection
(C-10)
(C-12)
(C-14)
(C-16)
(C-18)
16
14
12
10
8
6
4
2
0
32
30
28
26
24
22
20
18
16
Retention Time (min)

Table 9.

Phenol Oxidase Activity Using

Tyrosine and DOPA Substrates

Sample	Source of enzyme	Tyr/DOPA* activity
1	Unfractionated activation extract, 0.63 g/ml, pH 6.7	0.064
2	Unfractionated activation extract, 0.067 g/ml, pH 7.8	0.074
3	Band 2, pooled (see Fig.6)	0.084, 0.089, 0.120 ‡
4	Fraction 50, Fig. 6	0.13
5	Fraction 54, Fig. 6	0.012
6	Band 6', Fraction 15, Fig. 7	0.047
7	Band 7', Fraction 6, Fig. 7	0.027
8	Band 2, Fraction 4, Fig. 7	0.145
9	Band A, Fraction 4, Fig. 9	0.090
10	Band B, Fraction 20, Fig. 9	0.133, 0.117‡
11	Band C, Fraction 22, Fig. 9	0.057, 0.048‡

* This value was calculated from the activity per 100 micro liters using each substrate.

‡ Values were obtained from separate experiments.

composite band is fractionated into bands 6' and 7' (Samples 6 and 7), the difference in tyrosinase activities is still present, but smaller. The ordering of tyrosinase activities in Bands A, B, and C (Samples 9,10,11) suggests that these bands are qualitatively similar to 2, 6', and 7', respectively. Though the amounts of total phenol oxidase are different in corresponding bands, dilute and concentrated activating extracts seem to produce the same types of enzyme with respect to substrate activity.

Another set of experiments was conducted to determine the relative mono- and diphenol oxidase activity of detergent treated band 2 phenol oxidase. After 60 hours of incubation at 25°C, samples of band 2 enzyme, in 0.05 M tris buffer, pH 7.8, and either 5% NaDOC or 5% Triton X-100, were centrifuged at 100,000 x g (max. force) for 1 or 3 hours. The supernatants, or soluble enzyme, and resuspended pellets were assayed separately using tyrosine and DOPA substrate solutions. These results are shown in Table 10. The most interesting effect of 5% NaDOC is the enrichment for tyrosinase activity in the pelleted enzyme relative to the supernatant. A longer centrifugation time of 3 hours decreased the recoverable activity, especially with tyrosine as the substrate. The detergent actually seems to have enhanced the tyrosinase activity in the pelleted enzyme (116%). In contrast, Triton X-100 does not favor the assymetric distribution of tyrosinase activity between soluble and

Table 10.

Effect of Detergents on Relative Tyrosinase and DOPA Oxidase Activities of Phenol Oxidase

Sample	Centrifugation time (100,000 x g)	% DOPA Oxidase activity	% Tyrosinase activity	Tyr/DOPA*
5% NaDOC	-----	100	100	0.031
NaDOC supn't.	1 hr	56	13	0.0069
NaDOC pellet	1 hr	44	116	0.078
NaDOC supn't.	3 hr	69	20	0.0090
NaDOC pellet	3 hr	24	50	0.064
5% Triton X-100	-----	100	100	0.024
Triton X-100 supn't	3 hr	65	41	0.015
Triton X-100 pellet	3 hr	48	55	0.027

*This ratio was calculated from units of activity/100 µl, using each substrate.

insoluble fractions of the enzyme.

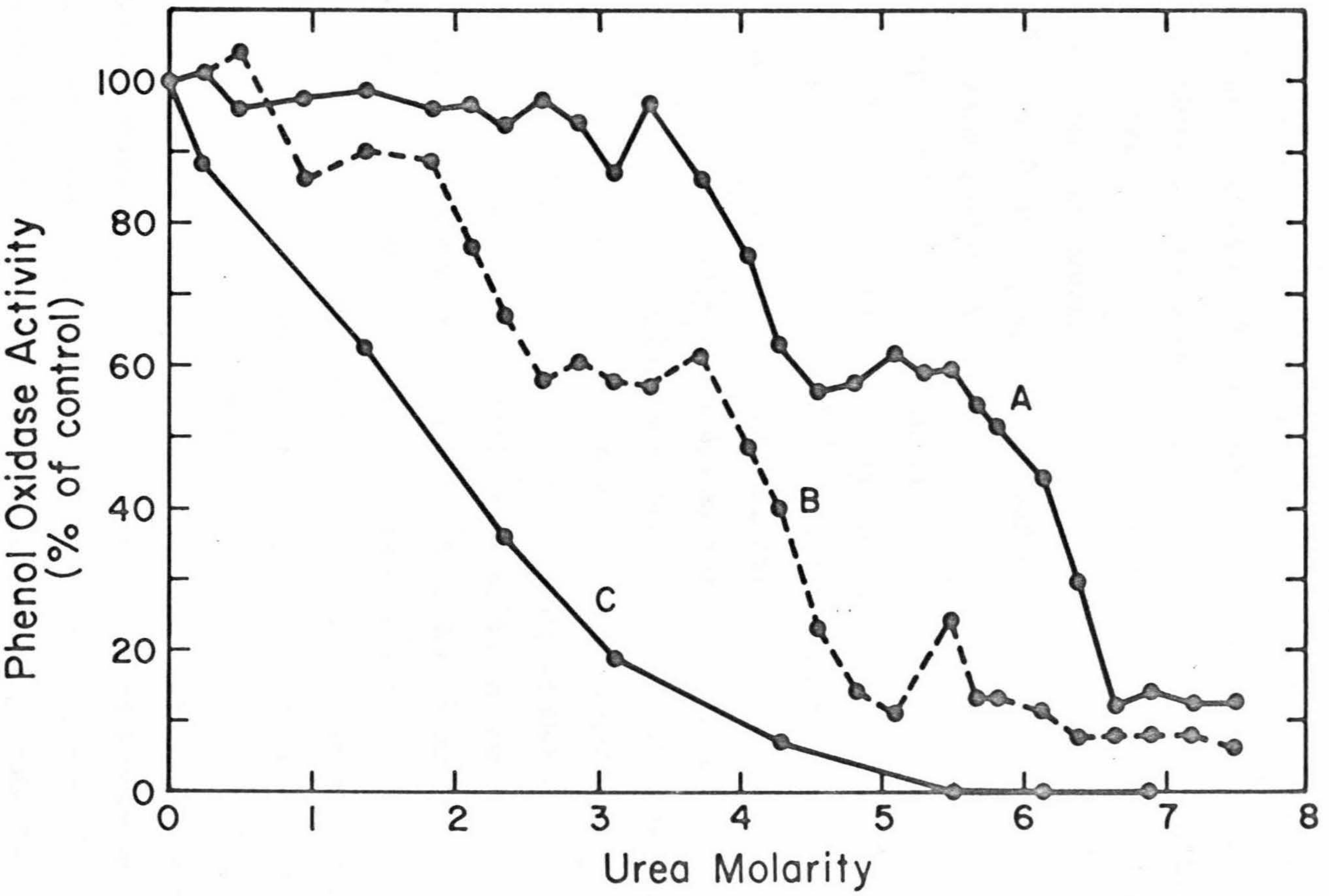
The detergents do seem to effect the tyrosine to DOPA oxidase ratio which is reduced to about one-third the value given in Table 9. While DOPA oxidase activity does not seem to diminish more rapidly than expected (5%/day), tyrosinase activity may be more sensitive to the incubation conditions. There was some indirect evidence that the buffer (tris, pH 7.8) and 25°C temperature were responsible for this, rather than the detergent.

The results indicate that NaDOC can alter phenol oxidase, when it is solubilized, so that it loses its tyrosine hydroxylase function. The tyrosinase activity, however, seems to remain in the pellet where it is still functional in conjunction with the remaining DOPA oxidase activity.

E. Activity and Denaturing Agents

The effect of various denaturing agents on phenol oxidase activity was observed during this work. As mentioned earlier, NaDOC and T X-100 enhance activity, with the former detergent showing a larger effect. Sodium dodecyl sulfate rapidly and irreversibly destroys activity, as do various organic solvents. Of more interest are urea and guanidine-HCl which have graded effects. Figure 15 shows the activity of phenol oxidase as a function of urea concentration. After full activation of this extract samples were added to pre-weighed quantities of urea and assayed for

Figure 15. The effect of urea on phenol oxidase activity. Phenol oxidase, prepared by procedure II, was activated for 4 hours at 0.1 g/ml in 0.1 M phosphate buffer, pH 6.3. One ml samples were then added to pre-weighed amounts of urea. Activity was measured within 1 min. of mixing (A) and after 25 hours (Curve B) at $0^{o}C$. Activities in curves A and B were obtained with DOPA as the substrate in 0.1 M phosphate buffer, pH 6.3. For curve C, the substrate solution contained the same urea concentration as the assayed sample. The activities have been corrected for dilution by the addition of increasing amounts of urea. This correction was only 3% of control activity at 7.5 M urea.



Phenol Oxidase Activity
(% of control)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Urea Molarity
A
B
C

activity in a substrate solution without urea. This gave a 25 fold dilution of the urea. Sample concentrations of urea above 3.5 M rapidly decreased enzyme activity in two steps (Fig. 15,A). After 25 hours incubation at $0^{o}C$, urea concentrations greater than one molar caused loss of activity in three steps (B). When urea was added to the substrate solution and activity measured after 25 hours, activity values were much lower (C). Comparison of curves B and C suggests that the stepwise nature of the former may be due to a partially reversible inhibition of phenol oxidase activity. Several phenol oxidase samples were incubated with urea (1.4,3.1,5.1 and 6.9 M) for 24 hours and dialyzed against phosphate buffer to remove the urea. The activity of these samples was, then, the same as in non-dialyzed controls. This implies that the activity reduction in curve B is due to irreversible effects on the enzyme, while the additional reduction in curve C is due to some reversible effect on the enzyme - substrate interactions.

Another experiment was performed using guanidine-HCl from 0.2 to 2.8 M. Similar results were obtained with a three step inactivation curve. Forty-five to 50% of control activity remained at 0.2 M guanidine and 3 to 5% at 2.8 M. This confirms that the effect of urea on phenol oxidase activity is a result of the way in which the enzyme is denatured and not a peculiar property of one denaturing agent.

4. DISCUSSION

A. Results

1. Aggregation and sucrose gradient separations. The studies of activation conditions and separation of active phenol oxidase on sucrose gradients have shown the diversity of aggregation states which are possible. The degree of aggregation depends primarily on the concentration of the activating extract, but may also be influenced by conditions of centrifugation and activation. The greater aggregation at higher activation concentrations can be explained as an effect on the activation process or as a secondary aggregation of the active enzyme units. In the former case, activation may result directly in the formation of a polymeric enzyme. Concentrated activation extracts may then produce higher order polymers. Alternatively, concentrated extracts may favor association of basic enzyme units. These two possibilities cannot be distinguished by the data presented here.

The difference in sucrose gradient sedimentation behavior of phenol oxidase produced at high and low activation concentrations could be a function of its composition as well as its aggregation state. In a given extract, dilution prior to activation should not affect the proportions

of components involved in the reaction. The data presented in Figure 3 suggest that this may be true over a 10 fold range of dilution. However, the decrease in activity recovery at very high dilutions indicates that the relative composition of the activation extract may be changed under these conditions. High dilutions could denature a phenol oxidase component or inhibit its production from a precursor, e.g., S from pre-S. None of the phenol oxidase prepared in the experiments presented here involved these very dilute activation conditions. It can then be assumed, for this discussion, that the primary effect of activation extract dilution was on the aggregation state of the phenol oxidase.

A hypothesis consistent with this view and with the sedimentation behavior of phenol oxidase is that the less aggregated, low density phenol oxidases, produced at low activation concentrations, are precursors of more highly aggregated phenol oxidases, such as band 2 enzyme. The low density enzyme (less than 1.10 to 1.18 g/ml) is composed of at least two components differing in substrate specificity. The lower density or smaller of the two components (band C, Fig. 9) has much lower tyrosinase activity than the other component (band B). These low density enzymes also have the highest specific activity (over 600 U/mg) of the forms analyzed (see Table 4). On the basis of density and substrate specificity it appears that concentrated activation conditions also produce these components (see Samples 4-7,

Table 9) but they represent only about 30 to 50% of the total enzyme activity.

High density phenol oxidases, like band 2 enzyme , can be considered higher order aggregates, perhaps containing some covalent cross links, of the low density forms. There are two lines of evidence to support this contention. In one case high density phenol oxidase was produced from lower density forms by sucrose gradient centrifugation; and, in the other, low density phenol oxidase was extracted from band 2 enzyme. When a dilute activation extract is centrifuged on a 45 to 64% sucrose gradient (as in Fig. 8) most of the activity remains at the top of the gradient with the occasional appearance of some activity (about 10%) at a higher density. When the same extract is centrifuged on a 5 to 64% sucrose gradient about 35% of the enzyme activity is found at higher densities (see Fig. 9) with about the same specific activity (470 U/mg) and substrate specificity (tyrosine/DOPA = 0.090) as band 2 phenol oxidase. This suggests that high density phenol oxidase components may be generated from lower density aggregates. In the second example, sodium deoxycholate solubilized up to 65% of the activity of band 2 phenol oxidase. From analogous experiments with deoxycholate treated activation extracts, the solubilized material seems to have a density of about 1.135 and a size distribution around 10^6 m w.

If band 2 phenol oxidase is simply a highly aggregated

form of basic enzyme forms, it is necessary to explain the changes in the amounts of high density forms, bands 1,2,3, and 4 (see Ref. 36), with age and genotype of the extracted animals. Another problem which arises is why these aggregated forms have discrete densities in sucrose gradients, rather than a wide range of density forms. A partial answer to the first problem can be obtained from the amino acid analysis data of Table 8. Bands 2 and 3, which have mean densities of 1.296 and approximately 1.250 g/ml, are very similar in amino acid composition. The density difference between them may not be due to protein composition but to non-protein constituents which contaminate these forms or to the difference in the amount of a minor protein which has a large effect on the packing and density of these aggregates. Another explanation which cannot be ruled out is that different proportions of phenol oxidase precursors somehow produce aggregated phenol oxidases of different densities.

The narrow density distribution of band 2 enzyme may be a characteristic of precipitated *Drosophila* proteins. This is suggested by the observation that, when *Drosophila* extracts are precipitated by heat or urea treatment, most of the protein formed a discrete, flocculent, equilibrium density band at 1.256 g/ml. This is about the same density as that of band 3 enzyme. The density positions of bands 1, 2, and 4 could be attributed to more specific precipitates, i.e., characteristic of some subset of the whole *Drosophila*

extract .

It is appropriate in this discussion of density variants to recall that the centrifugation process itself might affect the nature of the resulting phenol oxidases (see Ref. 36). One factor which is certainly important is the inelasticity of phenol oxidase in collisions with the centrifuge tube and perhaps with itself. If the enzyme hits the side wall of a centrifuge tube, due to radial sedimentation force in a swinging bucket, it tends to stay there. Also, if the force is great enough, pelleted phenol oxidase loses all but a few percent of its initial activity. This is probably the main reason for recovery of only 60 to 70% of the initial activity in the gradient shown in Figure 6. Another factor which may affect the phenol oxidase is the shear force resulting from transport through the gradient. The material in band 2 is composed of linear arrays and aggregates of large particles, up to 1 μ in diameter (see Fig. 3 in Ref. 36), which might be susceptible to such forces. It is also possible, as shown in Figure 9, that higher density aggregates may be produced by the movement of phenol oxidase through a sucrose gradient. None of these possibilities have been directly tested, but indirect evidence suggests that they may represent important characteristics of the *in vitro* enzyme.

2. Purity. The uncertainty in the origin of

phenol oxidase density bands makes it difficult to estimate their purity. The only way to achieve this is through complete dissociation of the enzymes in question and an analysis of the constituent parts. The partial dissociation and analysis on SDS acrylamide gels indicates the presence of contaminating proteins, or at least proteins other than A components, but this evidence is difficult to interpret (see discussion below). There is, however, indirect evidence on the purity of various forms of phenol oxidase. The addition of ^{14}C labelled E. coli protein to an activation extract showed that band 2 phenol oxidase is not significantly contaminated by general co-precipitation of the proteins in an activation extract. It could, however, still be contaminated by a few specific Drosophila proteins or lipids. Hemolymph proteins, which are present in high concentrations in protein extracts and which have low solubilities, are likely sources of contamination. Under conditions which inhibit phenol oxidase activation (1.5 M KCl or after appropriate ammonium sulfate fractionation) proteins precipitate out of a Drosophila extract for several days at $0^{o}C$ (58).

The similar amino acid analyses for band 2 and 3 could be taken as evidence for the purity of these phenol oxidases, but could also indicate a similar, highly heterogeneous protein composition. The specific activities of band 2 enzyme indicate a lower purity than low density forms

(470 vs 675 u/mg of protein or 30%) from dilute activation extracts. This decreased specific activity of band 2 phenol oxidase could also be explained by the proportions of high tyrosinase and low tyrosinase enzyme present. If bands B and C (Fig. 9) were mixed in equal parts they would give a specific activity and tyrosine to DOPA activity ratio equivalent to that of band 2 phenol oxidase. The theoretical maximum specific activity is not known but 675 U/mg represents a 50 fold purification from the total soluble proteins. Two percent of the protein is not unreasonably high, if this enzyme was a major constituent of imaginal epicuticle.

3. Composition. The chemical analysis of band 2 phenol oxidase indicated a protein containing lipid, hexoses, and phosphate. The molecular weight calculated from the amino acid analysis is not meaningful because the *in vitro* enzyme is probably composed of at least 2 or 3 different protein subunits including A_1, A_2 and S. The weight percentage of amino acids was 60 to 65% after acid hydrolysis. This value could be low by 10% or more due to destruction of amino acids. The phosphate, if all included in phospholipid, indicates about 6.5% by weight phospholipid. Hexose sugars account for 3% of the phenol oxidase weight. The additional 25 to 30% of the mass of this phenol oxidase may be accounted for by lipids containing no phosphate, amino acids destroyed in hydrolysis and possibly melanin or similar polyphenols produced by the phenol oxidase.

Some of the protein of band 2 enzyme may be covalently cross linked by oxidized phenols since only 75% is dissociated in concentrated formic acid. The presence of tyrosine in the activation mixture could account for this cross linking. The amount of cross linked or highly insoluble protein appears to vary with the source of phenol oxidase and method of preparation. This was evident in SDS acrylamide gel electrophoresis, for the same amounts of enzyme (determined from activities) produced detectable banding patterns at different amounts of sample. Also the amount of protein which was too large to enter the gel matrix varied considerably with different samples.

The results of SDS gels are complicated and difficult to interpret in terms of molecular composition of phenol oxidase. There is a suggestion that the active enzymes at least include the dissociated phenol oxidase components. The major protein triplet occurring at about 80,000 m.w. (see Figure 13) corresponds to the molecular weight of pure A_1. SDS treated preparations of A_1 give two closely spaced bands at about 77,000 on SDS acrylamide gel electrophoresis. The third component of the triplet may be A_2 which is known to be similar in size to A_1 (59).

The variation in number of bands on SDS gels (see Fig. 12) seems to be due partly to the protein concentration in each sample, as shown by comparing gels 1, 2, and 5 (with 24, 36, and 52 ug of protein, respectively). There are

several possible interpretations for the closely spaced multiple bands. They may be due to other phenol oxidase proteins, contaminating non-phenol oxidase proteins, an artifact of SDS binding to lipids, a proteolytic step involved in activation, or aggregation. Though none of these possibilities can be now excluded, some derive support from other observations. The possibility of contamination is great, especially for gels 4 and 5 since this enzyme sample had a very low specific activity (150 U/mg). However, the sample used for gel 2 had a high specific activity (over 600) and gave a similar banding pattern. Aggregation and the production of multiple bands on gel electrophoresis has been shown to occur with purified Drosophila alcohol dehydrogenase (60). In this case, SDS produced one subunit of 7,500 m.w. while urea plus SDS produced aggregates. It is also interesting that insoluble mouse tyrosinase was represented by one electrophoretic form in urea, but multiple forms in a detergent (11).

At best the SDS gel patterns imply that phenol oxidase may be dissociable into its component parts. The multiple band patterns might be due to variations in SDS binding to lipoproteins plus some protein contaminants. It is also possible that other phenol oxidase components, which are not necessary for activity, are represented. These might be responsible for structural integration into the cuticle or for modification of enzyme activity. Gels run under other denaturing conditions might eliminate some of the

uncertainties in interpreting these results.

B. General.

Phenol oxidase plays a major role in the production of cuticular structures by insect epidermal cells. The phenol oxidase deposited in cuticle determines its hardness and coloration. There is a great range of expression of enzyme function from the hard, but lightly colored mouth parts to darkly pigmented, hard sclerites or soft, flexible cuticle at articulating junctions. The particular role of cuticular phenol oxidase is determined in a mosaic fashion by the immediately underlying epidermal cells (61). The expression of cuticular hardness and coloration, then, is a part of the developmental program of these cells, as is bristle and hair formation and tracheal tube construction. In fact the morphological expression of these cells takes place primarily through the deposition of phenol oxidase rich epicuticle (62).

The study of phenol oxidase is, in this context, an important step in understanding the differentiation and morphological functions of epidermal cells. The _Drosophila_ phenol oxidase system is especially useful for the eventual application of genetic analysis to developmental processes. Many of the known _Drosophila_ mutants involve alterations in epidermal development or function. The first step in analyzing epidermal development through phenol oxidase is an analysis of the isolated enzyme. The information obtained on

isolated, _in vitro_, phenol oxidase can then be related to its _in vivo_ function and its regulation.

1. _In vitro_ phenol oxidase. The relationship between _in vitro_ and _in vivo_ phenol oxidases has not been clearly established. Indirect evidence suggests that inactive phenol oxidase precursors in the animal are used primarily for cuticle construction. For instance, in extracts of late third instar larvae, phenol oxidase activity results from A components in the hemolymph and S from the salivary glands (34), with possibly a small contribution from hemocytes. This potential activity is not needed for pupal case construction, but it is probably involved in imaginal cuticle synthesis. As Mitchell suggested (20), it is reasonable that the synthesis of a large amount of cuticular protein might occur during larval feeding stages and the precursors stored in an inactive form until needed for imaginal cuticle synthesis and melanization. Direct proof of this would require a demonstration that these phenol oxidase precursors are utilized in cuticle formation.

Indirect evidence has come from studies of potential activity during the pupal stage of wild and _straw_ type _Drosophila_ and by phenocopy induction (30). In wild type pupae, the peak of potential activity drops about 35% by 10 hours after puparium formation and remains at this level until the beginning of imaginal pigmentation (78 hours) when it again decreases. The _straw_ mutant, which has yellow

bristles and lightened body color, shows a much smaller reduction of potential activity at this later time, indicating decreased utilization of the available phenol oxidase for pigmentation. However, there is no decrease in activity between 10 and 78 hours when epicuticle synthesis may be taking place. It is possible that the decreased potential activity after puparium formation is due to incorporation of phenol oxidase into epidermal cell secretory granules which are later used for cuticle construction. The interpretation of these data is complicated by the lack of information on the factors effecting maximal activity in these extracts. If activation rate is considered rather than maximum activity there is a decrease for wild type at 40 and at 78 hours after puparium formation. For <u>straw</u> animals the latter decrease does not occur (see Fig. 3, Ref.30). This indicates that the S component, which seems to be rate limiting in activation, may be utilized more rapidly in the darkly pigmented animal, but during cuticle synthesis it is utilized equally in both types of pupae.

It is interesting to note that induction of blond bristles by heat shock has an optimum at 60 hours after puparium formation when epicuticle synthesis may be taking place. The result of this treatment is not only yellow thoracic bristles, but a change in potential phenol oxidase activity at the time of pigmentation, much like that seen for the <u>straw</u> mutant. Similar heating conditions prevent

enzyme activation in crude extracts by destroying S activity (59). Together, these results indicate that loss of potential phenol oxidase activity is related to the process of pigmentation and possibly to that of epicuticle formation.

In addition to these data there are several properties of active *in vitro* phenol oxidase which suggests its use in cuticle formation. The active enzyme is a highly insoluble lipoprotein which could serve as a component of the tough, water-proof epicuticle. It is also resistant to pronase digestion, which corresponds to the resistance of epicuticle and sclerotized endocuticle to digestion by moulting fluid enzymes.

Direct proof of the involvement of this phenol oxidase in cuticle synthesis could be obtained by studying the fate of precursor molecules from the hemolymph and salivary gland. This might be accomplished by radioactive labelling of purified samples of A_1 or S proteins or by immunological detection of these components. Until this evidence is available, it seems reasonable to assume that *in vitro* phenol oxidase is somewhat similar to the cuticular enzyme. The general properties of the *in vitro* product, as discussed below, can then suggest some limitations on the function and regulation of any *in vivo* phenol oxidases.

2. Composition and structure. It is now known that purified and homogeneous S, A_1, and A_2 proteins are sufficient to produce active phenol oxidase (see Introduction).

No combination of two of these components has yet produced phenol oxidase activity. The results of SDS acrylamide electrophoresis also suggests that the A components are major species in active phenol oxidase. The presence of S protein or other relevant species is uncertain. The bonds holding the subunits together, in the <u>in vitro</u> enzyme, are probably strong non-covalent bonds. Secondary quinone cross-linking may be responsible for the undissociable portions of the active enzyme. There is no good evidence for postulating other specific cross links, such as di- and trityrosine.

Listed below are the properties of phenol oxidase or its precursors which should be considered in a structural model.

1) A_1, A_2 and S are necessary and sufficient for activity.

2) A_1 and A_2 are probably present in the active enzyme.

3) A_1 produces an enzyme with tyrosinase and DOPA oxidase activity.

4) A_2 produces an enzyme with primarily DOPA oxidase activity.

5) The tyrosinase to DOPA oxidase activity ratio is variable over a 10 fold range.

6) A lower tyrosinase to DOPA oxidase ratio correlates with lower DOPA oxidase specific activity.

7) Active DOPA oxidase is more easily solubilized by sodium deoxycholate. Tyrosinase activity remains in the

insoluble part.

8) There may be at least 5 discrete activity states possible for active phenol oxidase, with DOPA as substrate.

It seems reasonable to assume that active phenol oxidase, like plant tyrosinase, has two copper atoms at the active site. In this case, one would be contributed by A_1 and the other by A_2. In the simplest model of phenol oxidase structure, A_1 and A_2 are joined together by one S molecule to form an active phenol oxidase. This, however, does not satisfy properties 5 and 7, above. It is possible to do this by assuming a much more complex arrangement of subunits in which active A_1-A_1 or A_2-A_2 complexes could form after activation. The insoluble nature of the enzyme could be explained by polymerization or aggregation of the A_1-S-A_2 subunits or polymerization of S units to which A_1 and A_2 are coupled. The complexity of this model can be increased by adding other subunits which modify the active enzyme or by more subunits at the active site. It is also possible that S activates A_1 and A_2 which can then combine in variable proportions. In this case S has a catalytic role and does not participate in the active complex.

Tests of these ideas can be performed with the purified A_1, A_2 and S components now available. The order of reaction, specificity and activity with varying proportions of components, and the physical nature of the resulting complexes can be determined using these purified proteins.

3. Properties of *in vitro* phenol oxidase. The mucolipoprotein nature of *in vitro* phenol oxidase and its insoluble, aggregated state make it a likely candidate for a component of the epicuticle. The properties of the active enzyme also suggest various ways of regulating *in vivo* phenol oxidase activity and the processes of cuticular pigmentation and hardening. It is worth considering in more detail the regulation of cuticular properties that occurs *in vivo* and the ways in which this could theoretically occur.

Larval cuticles of *Drosophila*, which have the normal components of epicuticle and chitinous endocuticle, are soft, flexible and unpigmented. In the late third instar this cuticle becomes the hardened puparium. In these cases regulation of hardness and, in some insects, melanization is fairly uniform over the cuticle, though there is some variation in each larval segment. In imaginal or adult animals, the epidermal cells show a much greater degree of differentiation by producing cuticles of varying hardness and pigmentation and complex structural modifications. In the adult cuticle the great morphological complexity arises from a mosaic determination of the epidermal cells. Most of the exoskeleton is hard and tanned with definite patterns of yellow or black pigmentation. Hairs and bristles are also sclerotized and pigmented, though pigmentation does not have to correspond with that of the

surrounding cuticle. Some cuticle, at the base of articulating appendages and bristles, is soft and flexible and some may be extremely hard, as in the mouth parts. The cuticular lining of tracheal tubes is a sheet of soft epicuticle with periodic spiral thickenings of hard cuticle. This structure is especially interesting for, when a tracheal tube is cut, the exposed ends rapidly blacken in a DOPA solution.

There are several ways in which the epidermal cells can regulate hardening and pigmentation of their corresponding areas of cuticle. The most obvious, suggested by the tracheal tube example above, is by regulation of the amount and type of substrate made available to the phenol oxidase. Mitchell (63) has pointed out that all degrees of hardness and pigmentation could be regulated primarily by the amount of substrate. Low amounts of substrate would cause cross-linking of cuticular proteins, but little coloration. Higher substrate concentrations would produce colored polyphenols. The type of substrate would regulate the color of the polymer (yellow, vs black or brown) and might also affect the type of cross linking. Bifunctional quinones resulting from a non-substituted diphenol would be an effective cross linker of proteins but would form only two dimensional polymers. Trifunctional quinones, as produced from DOPA, would produce highly branched and complex 3 dimensional polymers, as in indole melanin. The

relative importance of these factors in various types of cuticle has not yet been demonstrated. This could be accomplished by studies of the utilization of radioactively labelled substrates by epidermal cells.

Two other mechanisms of phenol oxidase regulation are of more interest in relation to the results of this thesis. This regulation could occur by changing the molecular composition and amount of active phenol oxidase secreted into the cuticle or by modification of phenol oxidase activity after it has been deposited in the epicuticle.

The results on substrate specificity of phenol oxidase have shown that the relative tyrosinase activity of the enzyme is variable over a wide range. DOPA oxidase activity is always much greater (6 to 60 times) than tyrosinase activity. Low tyrosinase enzyme deposited in the epi-cuticle, followed by a monophenolic substrate would result in a softer, less colored cuticle. This could also be accomplished by adding less phenol oxidase or lower specific activity phenol oxidase to the cuticle. These latter possibilities do not seem to be the mechansims employed by tracheal tube producing epidermis, but may be significant elsewhere, as in thin articulating membranes of the exoskeleton.

The studies of <u>in vitro</u> phenol oxidase activity and denaturing agents suggest that the enzyme can exist in at least five discrete and stable states of activity. This

phenomenon, which must be related to the organization which takes place during activation, indicates that an epidermal cell could regulate the activity level, possibly by varying the proportions of precursor proteins. Also the macromolecular environment (epicuticle composition) or some small diffusable molecules in the cuticle could determine the activity level of the phenol oxidase deposited there at one of these 5 levels. Studies of the effects of urea on phenol oxidase produced with purified S, A_1, and A_2 would be of interest to clarify this possible mechanism. It may eventually be desirable to define the phenol oxidase composition of a given area of cuticle. This would give a direct picture of the relationships among epidermal cell function, phenol oxidase structure, and cuticular properties. Because of the mosaic nature of cuticle and the insolubility of cuticular phenol oxidase, extraction of phenol oxidase and its analysis would not be practical. Histochemical means, using antibodies directed against phenol oxidase subcomponents, may give the needed information on the quality and quantity of phenol oxidase in the cuticle and also in the underlying epidermal cells.

REFERENCES.

1. Mason, H.S., in Advances in Enzymology (F.F. Nord, ed.) Vol. 16, p. 105, Interscience Publishers, N.Y. (1955).
2. Mason, H.S., Nature, 177:79 (1956).
3. Frieden, E., S. Osaki, and H. Kobayashi, J. Gen. Physiol., 49:213 (1965).
4. Bouchilloux, S., P. McMahill, and H.S. Mason.,J. Biol. Chem., 238:1699 (1963).
5. Fling, M., N.H. Horowitz, and S.F. Heinenann, J. Biol. Chem., 238:2045 (1963).
6. Wolfe, G.H. and D.L. Coleman, in Biology of the Laboratory Mouse (E.L. Green, ed.) p.405, McGraw-Hill Co., N.Y. (1966).
7. Moyer, F.H., Am. Zoologist, 6:43 (1966).
8. Seiji, M. and T.B. Fitzpatrick, J. Biochem., 49:700 (1961).
9. Menon, I.A. and H.F. Haberman, Arch. Biochem. Biophys., 137:231 (1970).
10. Rittenhouse, E., Develop. Biol.,17:351, 366 (1968).
11. Burnett, J.B., H. Seiler and I.V. Brown, Cancer Res., 27:880 (1967).
12. Burnett, J.B., T.J. Holstien, and W.C. Quevedo, Jr., J. Exp. Zool.,171:369 (1969).

13. Cottrell, C.B., in Advances in Insect Physiology (J.W.L. Beament, J.E. Treherne, and V.B. Wigglesworth, eds.) Vol. 2, p.175, Academic Press, N.Y. (1964).

14. Brunet, P.C.J., Ann. N.Y. Acad. Sci., 100:1020 (1963).

15. Pryor, M.G.M., in Comparative Biochemistry, (M. Florkin and H.S. Mason, eds.) Vol. 4, p.371, Academic Press, N.Y. (1962).

16. Hackman, R.H., in The Physiology of Insecta (M. Rockstein, eds.) Vol. 3, p.471, Academic Press, N.Y. (1964).

17. Mitchell, H.K., in Annual Review of Genetics (H.L. Roman, L.M. Sandler, and G.S. Stent, eds) Vol. 1, p. 185, Annual Reviews, Inc. (1967).

18. Andersen, S.O., Acta Physiol. Scand., 66:Suppl. 263, p.1 (1966).

19. Locke, M., in The Physiology of Insecta (M. Rockstein, ed.) Vol. 3, p. 379, Academic Press, N.Y. (1964).

20. Mitchell, H.K., U.M. Weber-Tracy, and G.Schaar, J. Exp. Zool., in press.

21. Mills, R.R., S. Androuny, and F.R. Fox, J. Insect Physiol., 14:603 (1968).

22. Stevenson, J.R. and T.Y. Adomako, J. Insect Physiol., 13:1803 (1967).

23. Karlson, P., Nature, 195:183 (1962).

24. Karlson, P., D. Mergenhagen, and C. Sekeris, Hoppe-Seyl. Z. 338:42 (1964).

25. Hackman, R.H., Proc. 4th Intern. Congr. Biochem., 12:48 (1959).

26. Yamazaki, H.I., J. Insect Physiol., 15:2203 (1969).

27. Hackman, R.H. and M. Goldberg, J. Insect Physiol., 13:531 (1967).

28. Lai-Fook, J., J. Insect Physiol., 12:195 (1966).

29. Horowitz, N.H. and M. Fling, in Amino Acid Metabolism (W.D. McElroy and B. Glass, eds.) p. 207, Johns Hopkins University Press, Baltimore (1955).

30. Mitchell, H.K., J. Insect Physiol., 12:755 (1966).

31. Lewis, H.W. and H.S. Lewis, Ann. N.Y. Acad. Sci., 100:827 (1963).

32. Rizki, M.T.M. and R.M. Rizki, J. Biophys. Biochem. Cytol., 5:235 (1959).

33. Ohnishi, E., Annot. Zoo. Jap., 27:188 (1954).

34. Geiger, H.R. and H.K. Mitchell, J. Insect Physiol., 12:747 (1966).

35. Ashida, M. and E. Ohnishi, Arch. Biochem. Biophys., 122:411 (1967).

36. Mitchell, H.K., U. Weber, and G. Schaar, Genetics, 57:357 (1967).

37. Waddington, C.H., Proc. Zool. Soc. Ser. A, 111:173 (1941).

38. Karlson, P. and A. Schweiger, Hoppe-Seyl. Z., 323:199 (1961).

39. Firtel, R.A. and G.B. Saul, J. Insect Physiol., 13:1197 (1967).

40. Ohnishi, E., J. Insect Physiol., 3:219 (1959).

41. Schweiger, A. and P. Karlson, Hoppe-Seyl. Z., 329: 210 (1962).

42. Ohnishi, E., K. Dohke, and M. Ashida, Arch. Biochem. Biophys., 139:143 (1970).

43. Preston, J.W. and R.L. Taylor, J. Insect Physiol., 16: 1729 (1970).

44. Mitchell, H.K., and U.M. Weber, Science, 148:964 (1965).

45. Meltzer, P., and D. Seybold, unpublished results.

46. Karlson, P. and H. Liebau, Hoppe-Seyl. Z., 326:135 (1961).

47. Sekeris, C.E. and P. Karlson, Pharmacol. Rev., 18:89 (1966).

48. Fogal, W. and G. Fraenkel, J. Insect Physiol., 15: 1437 (1969).

49. Fuzeau-Braesch, S., Bull. Biol. France and Belgium, 94:525 (1960).

50. Mitchell, H.K. and A. Mitchell, Drosophila Information Service, 39:135 (1964).

51. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, J. Biol. Chem., 193:265 (1951).

52. Waalkes, T.P., and S. Udenfriend, J. Lab. Clin. Med., 50:733 (1957).

53. Ames, B.N., in Methods in Enzymology (E.F. Neufeld V. Ginsburg, eds.) Vol. 8, p.115, Academic Press, N.Y. (1966).

54. Ashwell, G., in Methods in Enzymology (S.P. Colowick and N.O. Kaplan, eds.) Vol. 3, p. 84, Academic Press, N.Y. (1957).

55. Weber, K. and M. Osborn, J. Biol. Chem., 214:4406 (1969).

56. Good, N.E., G.D. Winget, W. Winter, T.N. Connolly, S. Izawa, and M.M. Singh, Biochem., 5:467 (1966).

57. Kieth, A.D., Comp. Biochem. Physiol., 17:1127 (1966).

58. Reynolds, J.A. and C. Tanford, J. Biol. Chem., 245: 5161 (1970).

59. Meltzer, P., Personal communication.

60. Jacobson, K.B. and P.Pfederer, J. Biol. Chem., 245: 3938 (1970).

61. Wigglesworth, V.B., The Control of Growth and Form, Cornell Univ. Press, Ithica, N.Y. (1959).

62. Locke, M., in Insects and Physiology (J.W.L. Beament and J.E. Theherne, eds.) p. 69, American Elsevier Publishing Co., N.Y. (1968).

63. Mitchell, H.K., Personal communication.

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
DFP	diisopropyl fluorophosphate
DOPA	3,4- dihydroxy phenylalanine
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
NaDOC	sodium deoxycholate
n m	nanometers
OD	optical density
PPO	2,5- diphenyloxazole
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
tris	tris (hydroxymethyl) aminomethane
TX-100	Triton X-100
Tyr	tyrosine
U	units of phenol oxidase activity
S. E. M.	standard error of the mean