A STUDY OF SOME OF THE ENZYMES INVOLVED IN THE SYNTHESIS AND USE OF TYROSINE IN *DROSOPHILA*

Thesis by

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I

Teaching the Ape to
Write Poems

They didn't have much trouble
teaching the ape to write poems:
first they strapped him into the chair,
then tied the pencil around his hand
(the paper had already been nailed down).
Then Dr. Bluespire leaned over his shoulder
and whispered into his ear:
"You look like a god sitting there.
Why don't you try writing something?"

Absences

James Tate

II

"...I knew that living was terrible and
that this was the answer to it. That
was when I learned that words are no
good; that words dont ever fit even what
they are trying to say at."

As I Lay Dying

William Faulkner

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I say thank you and good-bye (but hopefully not for long)

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ABSTRACT

Tyrosine is an integral component in sclerotization and melanization, two developmentally controlled processes which are necessary for synthesis and pigmentation of the insect cuticle. Studies were undertaken to investigate the means by which tyrosine arises and, also, the nature of the reaction whereby tyrosine is hydroxylated to yield dopa, the subsequent step in both of the above mentioned processes in Drosophila melanogaster.

It was found that phenylalanine is rapidly converted to tyrosine in vivo and the enzyme(s) responsible for the catalysis is readily extractable. The in vitro conversion is dependent upon the presence of tetrahydropteridine ($DMPH_4$), which is a cofactor in mammalian phenylalanine hydroxylase systems. Other common biological reductants are ineffective in this respect. The concentration of hydroxylase in the animal is developmentally regulated, the level building up during early third instar and peaking at puparium formation. Detectable quantities of enzyme are not found in pupae. The activity of the crude enzyme responds to the concentration of enzyme, substrate and $DMPH_4$ in a linear fashion (up to the point of saturation).

Tyrosine may give rise to dopa via two distinct enzymatic

means: tyrosinase or tyrosine hydroxylase. Drosophila are known to possess a very active tyrosinase system; however, this molecule also contains a dopa oxidase function which rapidly dehydrogenates dopa to yield dopaquinone, an irreversible step in melanization. In the absence of suitable regulation, this system does not allow for accumulation of dopa which is also necessary for sclerotization.

Crude extracts are capable of supporting the conversion of tyrosine to dopa in a $DMPH_4$ stimulated reaction. A high concentration of pteridine also serves to arrest dopa oxidase activity in vitro. $DMPH_4$ is an essential cofactor for mammalian tyrosine hydroxylase activity. The rates of accrual and utilization of dopa (by dopa oxidase) in crude extracts indicate that there are at least two synthetic pathways by which dopa arises. The ratio of the abilities to hydroxylate tyrosine and oxidize dopa varies as a function of the age of the animal, the highest ratio observed in early third instar larvae and the lowest at puparium formation.

The activity responsible for catalyzing the hydroxylation of tyrosine behaves in a manner similar to tyrosinase under a number of conditions. Pure tyrosinase, which is derived by sucrose gradient centrifugation, catalyzes a $DMPH_4$ stimulated hydroxylation reaction. Multiple forms of tyrosinase are derived in sucrose gradients and heterogeneity with respect to substrate utilization is observed in these species, a situation

which may have significance in a consideration of tyrosinase's involvement in supplying dopa for sclerotization. This possibility is discussed.

Since $DMPH_4$ plays such a crucial role in these reactions (stimulation of hydroxylation and inhibition of dopa oxidase activity), the nature of these involvements was investigated. There are some indications that the pteridine is not functioning to stimulate the hydroxylation reaction by serving as a non-specific reductant (reductants, such as, ascorbate and DPNH are known to stimulate the hydroxylase activity of pure tyrosinase), but may be serving in a more direct fashion with the enzymatic mechanism. The nature of this interaction is discussed.

Banded tyrosinase, using dopa as a substrate, catalyzes the synthesis of an unidentified compound. This reaction is stimulated by $DMPH_4$. The nature of this material and its significance to aromatic amino acid metabolism are discussed.

CONTENTS

INTRODUCTION

1. The importance of tyrosine to the metabolism of insects.

The multifunctional aromatic amino acid tyrosine plays a crucial role in the developmental metabolism of insects. In addition to the usual involvement of amino acids in protein synthesis, tyrosine has been implicated as being a key metabolite in cuticular synthesis, body pigmentation and neurological function (1). The ensuing discussions will emphasize the role of tyrosine in the former two processes.

A variety of insects have been studied with respect to deposition, hardening and pigmentation of the cuticle, and although it is apparent that the pathways differ among the many species studied, tyrosine and its derivatives are intimately involved in each one. This subject has been amply reviewed elsewhere (2,3,4,5,6,7), however, a brief outline of the basic mechanics of cuticle formation and pigmentation is in order.

Sclerotization. The cuticle can be divided into two regions: the inner, relatively thickened procuticle and the thin, outer epicuticle. The procuticle consists of protein and chitin, whereas the epicuticle, which is non-chitinous, consists of lipoprotein, wax and various diphenols. The epicuticle becomes hardened and colored, as does the outer region

of the procuticle, which now becomes termed the exocuticle, while the unreacted portion of the procuticle becomes the endocuticle. The most widely held belief is that the dihydroxyphenols (products of tyrosine hydroxylation) diffuse through the procuticle from the underlying epidermal cells out to the epicuticle where they are reacted upon by a phenol oxidase, which was laid down in the epicuticle earlier during the molt to form o-quinones. These highly reactive compounds react with free amino groups of neighboring proteins (and to some degree with ϵ amino groups of lysine) to yield a colorless hydroquinone derivative, which is oxidized by excess quinone to yield a quininoid protein. This derivative is able to form stable cross linkages with more free amino groups which results in the production of a lightly colored, insoluble polymer. Quinones must be in excess to allow for the synthesis of the quininoid protein. This product, which Pryor has termed sclerotin, is presumably responsible for the tan color and hardness of the cuticle.

Melanization. The above sequence of events, referred to as sclerotization, is distinct from melanization, which is also involved in imparting color (usually very dark) to the exoskeleton of insects. It was originally believed that hardening implied coloration. However, some insect structures, such as the pronatal extensions in Blabelus and the egg case of Periplaneta are hard, but colorless. Presumably, these

phenomena are the results of a sclerotization process without melanization occuring. In the context of the above model for sclerotization, the reactions take place in limiting amounts of quinone so that the colored quininoid protein does not accumulate.

Additional evidence for the existence of two distinct processes that are involved in cuticle formation and pigmentation comes from work done on the desert locust, Schistocerca. It was shown that an albino mutant, which is clearly defective in the melanization pathway, is still capable of forming a normal, rigid cuticle. This strongly suggests that melanization and sclerotization are separate, distinct processes (8,9).

Melanization is a multi-stepped process, the components of which have proven to be more tractable in the test tube than in the animal. The first step in the process, the oxidation of dopa to dopaquinone, is mediated by a phenol oxidase. The subsequent steps to melanin formation may require further participation of the enzyme, but these processes are poorly understood. Nevertheless, it is likely that indole-5,6-quinone arises, which then polymerizes to form melanin, a black insoluble substance. Mason has reported that the indole-quinone may also react with sulfhydryl groups of neighboring proteins (10). The contribution of the protein derivative versus the melanin polymer to the final darkly colored product

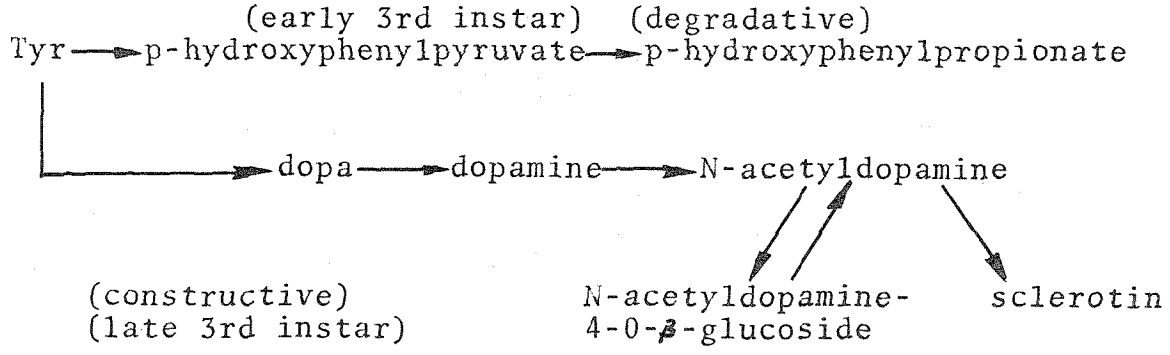
is undefined.

Although these processes of sclerotization and melanization are distinct, they are very similar in their requirements for substrates (phenolic derivatives) and enzymes (phenol oxidases). Whether or not two distinct phenol oxidases are required is a matter for interesting conjecture. This question will be addressed further in the section on tyrosinase (phenol oxidase).

The nature of the diphenols involved in sclerotization. Extensive studies have been performed to identify the nature and origin of the diphenols involved in sclerotization. In the blowfly Calliphora, it was noted that the level of tyrosine in the hemolymph rose to its highest concentration prior to puparium formation and then dropped precipitously once the puparium was made (2). It was later demonstrated that when C^{14}- tyrosine was injected into larvae sometime before pupation, label was found in the cuticle. Similar results were obtained when labelled dopa was administered (11).

Karlson and co-workers subsequently demonstrated that the immediate precursor to deposition of label is N-acetyl-dopamine (12). A tyrosinase was purified whose best substrate was this compound and which had no measurable activity with monophenols (13). When N-acetyl-dopamine labelled in the acetyl group was administered, label was found in the cuticle, suggesting that the intact molecule was incorporated. By

pursuing these sorts of experiments, along with demonstrations of some of the enzymatic activities involved in the pathway, these workers postulated the following scheme for tyrosine metabolism in Calliphora (12):



The molting hormone ecdysone is necessary for the constructive phase to occur. Apparently the hormone acts on controlling the action of dopa decarboxylase (12).

Sekeris and co-workers have also offered evidence that N-acetyl-dopamine plays the same role in sclerotization of the adult cuticle (14). The metabolism of tyrosine and dopa appear to be the same at eclosion as during puparium formation and the appearance and disappearance of dopa decarboxylase are also similar. Seligman, et al. offered a pertinent criticism of some of these experiments. They argued that, since the endogenous pool of dopa is so diminutive, injection of any amount of dopa will seriously disturb the natural pool size, resulting in abnormal metabolism. Studies done with tyrosine are more meaningful because of its larger pool size (15).

This pathway for tyrosine metabolism is by no means a

generalized process for all insects:just as clearly as N-acetyl-dopamaine is the immediate precursor to sclerotin in the blowfly puparium and adult cuticle, are the data showing that protocatechuic acid (dihydroxybenzoic acid) serves the same role in sclerotin formation in egg capsules of Blatta and Periplaneta (3,16,17,18,19,20).

If labelled tyrosine is injected into appropriately staged animals, label is recovered in the cuticle in the form of protocatechuic acid. Unfortunately, the enzymology of the steps from tyrosine to this compound is unknown. Pryor criticized these experiments (apparently his own included) based on the fact that, if these compounds are involved in sclerotin synthesis, they should not be so readily extractable (17). It will be recalled from the previous discussion that the diphenols, after oxidation, react readily to form covalent bonds with amino groups of surrounding proteins. It would be more meaningful if the labelled material were isolated from the hydrolysate of the sclerotin.

There is an additional complication in the Drosophila pathway. Mitchell and Lunan have described the occurrence of tyrosine-O-phosphate (21,22). The kinetics of its appearance and disappearance, namely that it accumulates to its highest level at late third instar and drops precipitously upon puparium formation, suggest that it is involved in cuticle formation. This compound is not a substrate for tyrosinase, nor does it inhibit the catalytic activity of this enzyme.

Its time of disappearance coincides with a rise in inorganic phosphate, suggesting that a phosphatase is also involved, thereby freeing the tyrosine for participation in further reactions. It is likely that tyrosine-O-phosphate is a storage form of tyrosine.

Seligman, et al. came to the same conclusion in their studies on the fly, Sarcophoga (15). It is reasonable to believe that such storage forms do exist, for huge amounts of tyrosine must be readily available when it is time to lay down the cuticle. Because of the relatively high solubility of tyrosine-O-phosphate, it would be an effective storage form for the less soluble tyrosine. However, these investigators cannot account for all the necessary tyrosine to be in the form of tyrosine-O-phosphate, and they have some indication that a tyrosine rich protein can also serve such a storage function.

Lunan and Mitchell further explored the chemistry of cuticle synthesis in Drosophila and demonstrated that there exists a developmentally regulated dopa decarboxylase, which is responsible for the synthesis of dopamine, and whose peak of activity appears at puparium formation (22). Presumably, a transacetylase is also active to yield N-acetyl-dopamine, which then diffuses out to the epicuticle. These workers also reported that N-acetyl-dopamine is present in prepupae.

It should be pointed out that in order for a dihydroxyphenol to be an effective substrate for tyrosinase in the sclerotization reaction, its structure must be such that, upon oxidation, it cannot rapidly cyclize to the indole-quinone, a step which would effectively shunt the molecule to the melanization pathway. Compounds, such as, N-acetyl-dopamine, protocatechuic acid and 4,5-dihydroxyphenylacetic acid cannot cyclize to the indole-quinone, whereas dopa and dopamine readily yield this product upon oxidation.

One fact which clearly emerges from these diverse studies on cuticle synthesis and pigmentation is that tyrosine and its derivatives are absolutely necessary constituents for these occurrences.

The purpose of this thesis is two-fold: firstly, to investigate the synthetic means by which tyrosine arises in Drosophila larvae; and, secondly, to elucidate the means by which tyrosine is converted to dopa that is to be used eventually for sclerotization. Part 2 of the Introduction relates information germane to the synthesis of tyrosine and part 3 deals with the nature of the hydroxylation of tyrosine to yield dopa.

2. The conversion of phenylalanine to tyrosine.

The existence of phenylalanine hydroxylase in insects

Because of the obvious importance of tyrosine in the development of insects it is of interest to study the manner in which tyrosine is accumulated.

Phenylalanine is a dietary requirement for insects and, if the animal ingests enough of this amino acid, tyrosine may be deleted from the diet (25). Unlike the microorganisms, insects are incapable of synthesizing the aromatic ring.

From these simple observations on nutritional requirements, it would appear that phenylalanine is capable of giving rise to tyrosine. Bricteux-Gregorie, et al. (23) and Fukuda (24, 26) independently demonstrated that tyrosine did arise from phenylalanine in the silkworm, Bombyx. C^{14}-phenylalanine was injected into larvae which were allowed to develop and subsequently spin the silky cocoon case. The fibers of this case contain a tyrosine rich protein, which, in this case, when isolated, hydrolyzed and analyzed were shown to contain radioactive tyrosine. In the experiments performed by Bricteux-Gregorie, et al., only the carboxyl carbon was labelled, which

suggested to them that the conversion is direct, probably via a simple hydroxylation. Neither set of experiments appears to deal with the possibility that tyrosine synthesis could be mediated by a symbiont residing in the silkworm.

Similar results were derived by injection of C^{14}-phenylalanine into adult cockroaches (27). Extensive conversion to tyrosine was observed in various aged adults. Radioactive benzoyl-β-D-glucoside was also formed in fully tanned adults, but not in animals that were 24 hours past ecdysis. Neither the pathway by which this compound arises nor its metabolic significance is known. Aposymbiotic animals were capable of supporting both tyrosine and glucoside syntheses.

Cell free extracts derived from larvae of many different species of insects contain components capable of converting phenylalanine to tyrosine (28). Belzecha and co-workers detected apparent hydroxylase activity in larval extracts of all species checked, whereas the two pupal extracts assayed showed no such activity. In light of later developments in hydroxylase chemistry, it is apparent that their assays were performed under sub-optimal conditions. However, the data are probably valid in a qualitative sense.

One might conjecture that the synthesis and function of phenylalanine hydroxylase are strictly regulated during development of the insect. We have learned from a number of microbial enzyme systems that the activity of the first step

in a biosynthetic pathway is often susceptible to modulation by various metabolites, thereby permitting the organism to metabolize as efficiently as possible. Since the product of the first step in the biosynthetic pathway to cuticle formation, tyrosine, must be available in large quantities at the time of each molt, molecular provisions must be made to ensure that the hydroxylase activity is great enough to furnish the animal with an ample supply of tyrosine. A further consideration that should be taken into account is the contribution of tyrosine via the hydroxylase reaction as opposed to that arising from the natural diet of the animal. No doubt the latter source does contribute, and the hydroxylase could be present to ensure that there is an ample supply for cuticle synthesis.

Since there is such a paucity of information concerning the nature of phenylalanine hydroxylase in insects, I must resort to a discussion of what is known about the enzyme in other systems.

Phenylalanine hydroxylase activity in other organisms

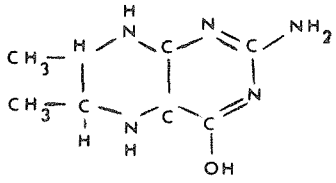
The rat hepatic system The most extensive studies performed on phenylalanine hydroxylase have been done with the rat liver system, most notably by Kaufman and his collaborators. He has recently presented a thorough review of the subject (29).

Research carried out during the last two decades on

phenylalanine hydroxylase has revealed that a large number of components are necessary for what appears to be a simple reaction to occur.

In 1957, Kaufman demonstrated that at least two enzymes, phenylalanine, molecular oxygen and a reduced pyridine nucleotide are necessary for the hydroxylation reaction to take place (30).

Subsequent investigations demonstrated the need for another component in the system, namely a tetrahydropteridine (31). A number of different pteridines were synthesized and assayed for activity. 2-Amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine and 2-amino-4-hydroxy-6-methyl-tetrahydropteridine were shown to be very active. The structure of the former is shown here:



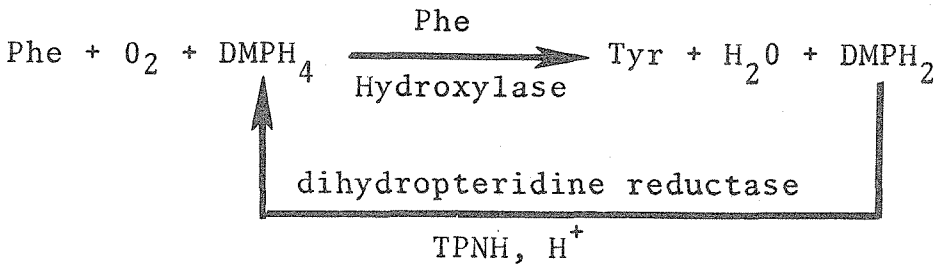
It was later demonstrated that the structure of the cofactor indigenous to the rat liver is dihydrobiopterin, or 7,8-dihydro-2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-ertyro)]-pteridine (32). Also, it is of special interest to note that an extremely active pteridine can be isolated from Drosophila (33). This compound, termed sepiapterin, is structurally

very similar to biopterin.

Pteridines function by reducing the unincorporated oxygen atom resulting from the hydroxylation reaction and are themselves oxidized in the process. If adequate quantities of pteridine are used, the system no longer requires TPNH (the reduced pyridine nucleotide referred to previously) and one of the two protein fractions included on the initial list of requirements (34). TPNH serves to reduce the dihydropteridine generated in the hydroxylation reaction and one of the protein components mentioned above catalyzes this regeneration step.

Experiments utilizing O^{18} demonstrated that the incorporated oxygen atom is derived from molecular oxygen, not water (35).

By way of summary, a plausible model for the rat liver hydroxylase system can be presented thusly:



Developing a scheme for purification of the rat liver enzyme proved to be a difficult matter. The inherent complexity of the system contributed to the problem and, even

after all of the components necessary for optimal efficiency had been defined, the enzyme itself proved to be very unstable.

Kaufman and co-workers have managed to derive preparations that are about 90% pure (36). Based upon data adduced from gel filtration, acrylamide gel electrophoresis and electrophoresis in sodium dodecylsulfate, Kaufman postulated that the enzyme exists in two isozymic forms, each one capable of existing (and presumably functioning) as a monomer of molecular weight of 51-55,000 daltons, a dimer of molecular weight of 110,000 daltons and a tetramer of 210,000 daltons.

Ferrous ion chelators are potent inhibitors of phenylalanine hydroxylase (29). However, as Kaufman noted, the chelators which were used in the experiments, such as o-phenanthroline, hydroxyquinoline and 2,2'-dipyridine, are all heterocyclic compounds which bear structural resemblances to pteridines. Hence, these reagents could be perturbing the pteridine chemistry of the reaction. The combination of cysteine and EDTA is an effective inhibitor of hydroxylase activity. The cysteine could be necessary to reduce a critical disulfide bond, consequently allowing the EDTA to react with the freshly exposed Fe^{+2}. It would seem that if the pure enzyme were available, it would be possible to settle unequivocally the issue of whether or not phenylalanine hydroxylase requires ferrous ion for its activity. This is a critical question because any discussion concerning the

chemical mechanism of the hydroxylation reaction must take this ion into account.

Data derived from kinetic analyses of the hydroxylase system indicate that the reaction is of the 'rapid equilibrium random' type (29). It is likely that a quaternary complex made up of enzyme, oxygen, pteridine and phenylalanine is formed. There had been conjecture that the enzyme first had to be reduced by the pteridine. A search for the hypothetical reduced enzyme was undertaken employing stoichiometric amounts of enzyme and all possible combinations of substrates which might be requisite for this reduction, but none was detected.

The actual chemistry of the reaction has not been unequivocally defined. Through use of phenylalanine labelled in the 4 position with either tritium or deuterium, it was shown that the original 4 position substituent migrates to the 3 position via formation of a cationoid intermediate in the reaction (37).

A number of experiments have indicated that the nature of the reaction catalyzed by the hydroxylase is dependent upon the physical state of the enzyme. The individual states, one being an associated form of the other, have not been physically isolated and characterized, but merely have been inferred from the quality of the reaction catalyzed under

various assay conditions.

The type of reaction observed, and therefore, the state of enzyme inferred, is dependent upon enzyme concentration, pH, ionic strength, temperature, substrate and cofactor analogs and a proteinaceous stimulating factor (38,39, 40).

The two types of enzymes which apparently exist have different Km's and Vmax's (29). Additionally, these two enzymes vary in their abilities to catalyze efficiently coupled reactions between phenylalanine hydroxylation and concomittant oxidation of tetrahydropteridine (38). Under certain conditions, more pteridine is consumed than phenylalanine is hydroxylated.

It was mentioned previously that the hydroxylase can exist as either a monomer, dimer or tetramer. There is also evidence that there are isozymic forms of the enzyme. These various states may manifest diverse catalytic properties referred to above, however, they have not been tested in this fashion. Even though the conclusions concerning regulation derived enzymologically appear to be consistent, unequivocal conclusions await isolation and careful characterization of the different catalytic forms inferred.

Tourian has recently presented evidence that the hydroxylase is capable of being activated by phenylalanine at a second site on the enzyme molecule (41). The s value of the

enzyme shifts dramatically upon activation, going from a form corresponding to a molecular weight of 110,000 daltons to one with a molecular weight of 210,000 daltons. These values are very consistent with the molecular weights derived by Kaufman. Unfortunately, the preparations used here were not of high purity so that the conclusions on the nature of the molecular interactions must await results from similar experiments on more homogeneous material.

A number of potentially significant interactions between various molecules and rat liver hydroxylase been described. Kaufman reported that norepinephrine inhibits activity (29).

Bublitz recently demonstrated that this inhibition can arise in a number of different ways (42). It appears that the catecholamine can be oxidized to o-quinones since ascorbate antagonizes the inhibitory effect and also because oxygen is a requirement for the inhibition to occur. o-Quinones are capable of reacting with free amino groups of proteins, thereby potentially interfering with catalytic activities of the enzymes. There is also an ascorbate insensitive inhibition of hydroxylase activity by norepinephrine. The catecholamine acts in a competitive capacity with pteridine and in a non-competitive manner with respect to phenylalanine.

Since norepinephrine is derived indirectly from phenylalanine, it is conceivable that this compound is exerting an

end product inhibition effect on the hydroxylase, a process which may have implications in regulation of nerve cell function. Of course, since the source of enzyme in these studies is liver tissue, reservations must be made concerning these results, however, it is possible that a similar system is operative in nerve tissue.

There are some indications, albeit very indirect, that phenylalanine hydroxylase is capable of catalyzing the hydroxylation of tryptophan to 5- hydroxytryptophan. Renson, et al. demonstrated that partially purified hydroxylase from rat liver was capable of catalyzing the hydroxylation of both phenylalanine and tryptophan (43). The cofactor requirements were the same and L-phenylalanine acted as a competitive inhibitor of tryptophan hydroxylase activity.

Freedland, et al. offered similar evidence using even cruder material (44). If excessive amounts of phenylalanine were included in the rat's diet, phenylalanine hydroxylase and tryptophan hydroxylase activities were inhibited to the same degree; also, excessive tryptophan induced both activities to a similar extent. However, addition of various reagents, such as Fe^{+2}, ascorbate and Cu^{+2}, had disparate effects on the two enzymatic activities, indicating to these investigators that more than one active site (but still only one enzyme molecule) was involved (45).

Renson, et al. feel that this tryptophan hydroxylase activity is of limited physiological significance (46). The only organ in which either activity can be detected is the liver, although serotonin (the decarboxylated derivative of 5-hydroxytryptophan) is wide spread. One would be forced to postulate that 5-hydroxytryptophan is synthesized in the liver and then transported, via the circulatory system, to organs containing the decarboxylase activity. This seems rather unlikely and, in fact, 5-hydroxytryptophan cannot be detected in the blood stream.

Some of these data are suggestive, however, to settle the question unequivocally, more homogeneous enzyme preparations must be employed. It would be of interest to know if Kaufman's purified hydroxylase is capable of hydroxylating tryptophan. Of course, negative data in this sort of experiment are not conclusive because a critical factor or subunit may be lost during the purification. Similarly, if it were found that the purified material did contain this new activity, gratuitous binding of a distinct tryptophan hydroxylase molecule would have to be ruled out.

Attempts to elucidate the nature of developmental regulation of synthesis and function of the rat liver hydroxylase have yielded contradictory results. Part of the problem is due to the number of variables in the system; conditions have

to be created so that the quantity of the particular component being measured is the only rate limiting factor in the assay.

Kaufman and co-workers have presented evidence that phenylalanine hydroxylase is not present in the fetal rat liver until the day before birth (47). However, the pteridine and pteridine reductase are present, albeit at very low levels, during fetal life. These investigators suggest that these components are being used for other hydroxylations. In newborn rats, if the entire system (both enzyme and co-factor) is assayed, 50-70% of the adult activity is observed in the newborn (48). The cofactor is the limiting component in the young rats; if both newborn and adult livers are assayed in an excess of cofactor, the levels of hydroxylase are nearly equal.

Contrary to these results are those presented by McGee, et al. Using what appears to be an adequate assay system, they demonstrated that the newborn rat has only 20% of the hydroxylase activity of an adult (49). At the second week of postnatal development, this figure is 40%. Cortisol administration at this period increases in vitro measured activity by a factor of two. This hormone has no effect on adult levels of hydroxylase.

Results even more at odds with those published by Kaufman have been presented by Tourian and colleagues (50). There are three peaks of activity found during embryonic life, the highest level achieved 23 days past conception. Furthermore, the newly born animals have only a fraction of the activity seen in the adults under their assay conditions, which do seem adequate. Additionally, various hormones are incapable of drastically altering the levels at certain periods of development. Tourian feels that he is more careful in staging his animals with respect to age than was done in previously reported experiments, however, this reason does not seem adequate to explain the gross discrepancies in the data.

Although the results from these experiments are inconclusive, it is apparent that control mechanisms are operative on synthesis of the components of the phenylalanine hydroxylase system in rat liver.

Phenylalanine hydroxylase in other organisms

Very little biochemistry has been done on the phenylalanine hydroxylase system in other organisms. Wherever it is found, the basic substrate, cofactor and cofactor regeneration system requirements are similar to the rat liver case.

The enzyme isolated from Pseudomonas species has been

partially purified and it was shown that it shares many properties with the rat liver system (51). It has an apparent molecular weight of 60,000 daltons, which, as Kaufman has noted, is about the same as for the rat liver monomer. It differs from the rat enzyme in that it is subject to metal ion activation, although this activation is nonspecific and has no apparent effect on the enzymology of the system.

Extracts of E. coli, S. faecalis and yeast are without activity (52).

Phenylalanine hydroxylase has been purified extensively from the liver of the monkey, Macaca isus (53). Two distinct protein molecules are required for full hydroxylase activity. One of the components (E) has some activity and is stimulated four fold upon addition of the other component (Y), which also has some slight activity itself. The completely active molecule, upon gradient and chromatographic analysis, displays multiple forms of activity. A peculiar result is that the rate of the reaction under these conditions is already falling off after 5 minutes. This phenomenon should be investigated, for the enzyme (or substrate or cofactor) could be inactivated, leading to spurious conclusions concerning the nature of the system. Although the enzyme has been purified almost to homogeneity, very little is known of its nature.

Studies done on the human liver phenylalanine hydroxyl-

ase system have additional significance since there is a large body of evidence that the genetic lesion responsible for phenylketonuria (PKU) is reflected in the patient's inability to convert phenylalanine to tyrosine. In vivo assays show that in some cases, there is no conversion, whereas with others, it is slight compared to appropriate controls (54, 55).

In two cases studied, liver extracts derived from autopsies were shown to be devoid of the ability to catalyze the conversion (56). Of course, it should be apparent that the absence of any of the factors would account for this effect.

Kaufman has demonstrated with a few PKU liver samples that the cofactor was present, implying that the defect was caused by a complete absence of phenylalanine hydroxylase or by an inefficient form of it (57).

Friedman and Kaufman have shown with normal human livers that the hydroxylase qualitatively shares many properties with the rat liver system (58). The human enzyme may exist in two forms: the soluble one and a particulate one (59). The latter may be converted to the former by either treatment with detergents or by sonication. The two forms are quite similar with respect ot their catalytic properties.

Additionally, the hydroxylase, cofactor and reductase system were present in some fetal samples studies (60).

Unfortunately, because of the lack of a readily available source of material, little work has been done on the human liver system. Such research is important in any consideration of chemotherapeutic treatment of PKU patients. As previously mentioned, the inability to catalyze the conversion of phenylalanine to tyrosine can be the result of a deficiency in any one of the components. From the definitive work on rat liver hydroxylase, molecular controls upon the activity are operative, therefore, abnormalities in regulation can also account for deficiencies in the reaction.

3. The conversion of tyrosine to dopa

I shall next take up a consideration of the initial reaction in the pathway that leads to cuticle formation, namely the hydroxylation of tyrosine to dopa. It will be recalled from the previous discussion on the chemical steps involved in cuticle formation that, even though the ultimate reactants in sclerotization for various species differ (that is, N-acetyldopamine in some, diphenol carboxylic acids in others) it is clear that each is derived from dopa.

Tyrosine may give rise to dopa via two possible enzymatic means: tyrosine hydroxylase (E.C. 1.14.3.1) or tyrosinase (E.C. 1.10.3.1). Both of these enzymes are capable of catalyzing the ortho hydroxylation of tyrosine, however, it is not known which enzyme is involved in the in vivo situation in insects. The remainder of this Introduction will be concerned with describing the nature of both of these enzyme systems, with emphasis on their distinguishing properties and potential significance to insect metabolism.

Tyrosinase.

The existence of tyrosinase in insects and something of its nature have been known for almost a half of a century. This one enzyme is capable of catalyzing both the ortho hydroxy-

lation of tyrosine and the dehydrogenation of dopa to yield dopaquinone. Tyrosinase may also be involved in subsequent cyclizations and oxidations in the pathway to melanin. There have been no instances reported where the activities (monophenol oxidase or cresolase and dopa oxidase or catecholase) have been physically separated from one another. This situation holds true for all tyrosinase systems studied to date and has been reviewed extensively (61,62,63).

This apparent association of two distinct catalytic activities in one enzyme complex presents obvious problems, for, in the in vitro case, under normal reaction conditions, the formation of dopaquinone is extremely rapid, thereby not allowing the newly synthesized dopa to accumulate and participate subsequently in the sclerotization reactions. Hence, if tyrosinase is the natural mediator of the tyrosine hydroxylation reaction, means must be provided to ensure that enough dopa escapes from the dopa oxidase reaction to be available for sclerotization. The following discussion will center on the nature of tyrosinase and the hydroxylation reaction which it catalyzes and potential control mechanisms which could afford for dopa accumulation.

The most definitive chemistry done on this enzyme system utilizes material obtained from one of three sources: the common mushroom, Neurospora and mammalian melanocytes. A discussion of these systems immediately ensues, followed by a consideration of insect tyrosinases.

Non-insect systems. Tyrosinase from these three sources have been extensively purified, and in no single case, have the monophenol and diphenol oxidases been dissociated from one another. However, preparations have been derived that vary greatly in their relative abilities to catalyze the two reactions, or as it has been traditionally described, there is variation in the cresolase to catecholase ratios. Under certain conditions, preparations rich in cresolase activity can be derived, while utilization of different purification techniques results in high catecholase activity (63,64,65,66,67, 68). Hence, in these systems, heterogeneity exists in the tyrosinase population with respect to relative use of substrates.

Horowitz, et al. screened a number of different strains of Neurospora and found at least 4 distinct types of tyrosinase based on thermostability and migration upon paper electrophoresis (69). These variations are genetically controlled.

The Neurospora enzyme has been purified to crystallinity (70). The molecular weight of the active enzyme, which undergoes a rapid association-dissociation equilibrium, is 32,000 daltons. One molecule of enzyme possesses one copper atom (isolated in the cuprous state).

It should be inserted here that a few investigators believe that the hydroxylation of monophenols occurs non-enzymatically (62). Briefly, the dopaquinone resulting from dopa oxidation is responsible for the hydroxylation. As will

be seen shortly, when a monophenol is used as substrate, trace amounts of dopa (which can be present as a contaminant in the substrate) greatly stimulate the reaction.

This non-enzymatic hypothesis seems unlikely in light of what is known of the nature of the reaction. Arguments against it have been neatly summarized by Lerner (63) and more recently by Mason (71). One of the most compelling arguments is that independently prepared ortho-quinones are unable to support hydroxylation of monophenols sans enzyme. Another is that the oxidation of tyrosine is optically specific (D-tyrosine is reacted upon slowly) - it is improbable that a non-enzymatic mechanism possesses this specificity. Additionally, the fact that preparations with varying cresolase to catecholase activities can be isolated lends creedance to the notion that discrete monophenolase activities do exist.

An obvious peculiarity of the cresolase reaction is the initial lag period before any product is accumulated. This situation is not observed in the catecholase reaction. In all except one case studied, this phenomenon has been observed. Kark anis and Frieden obtained an extremely pure preparation of mushroom tyrosinase that did not exhibit this lag (72). However, when it was treated with the side fractions from the last purification step, the lag returned and the rate of reaction diminished.

The duration of the lag is subject to a number of variable

(64,73). Also, high concentrations of monophenol at a fixed enzyme level prolong this inactive period. (68,73).

The entire lag can be obviated by either addition of trace amounts of dopa or of larger quantities of reducing agents, such as, ascorbate and DPNH (74,75,76,77). The modes of action of these compounds in abolishing the lag are not well defined. Dopa itself is a strong reducing agent, so the two effects may be related. Only trace amounts of dopa are required because the enzyme generates more reductant with time. It is also possible that dopa is acting in a manner unique from the reducing agents, perhaps by allosteric transformations.

When either ascorbate or DPNH is used, dopa accumulates even though the dopa oxidase function is present and active, the dopaquinone being reduced back to dopa as soon as it is formed. Krueger (75,76) and Kendall (74) believe that ascorbate does not merely function by favoring dopa accumulation (and thereby accounting for the effect), but is involved in a more direct fashion with the enzymatic mechanism. Besides the abrogation of the lag period, ascorbate also increases the rate of oxidation of tyrosine, something which is not seen if trace amounts of dopa are used in place of ascorbate (78). Krueger argues further that if the ascorbate were merely favoring accumulation of dopa, there should be a lag period when dopa is not present - as noted, such a lag does not exist. Additionally, there is a consumption of molecular oxygen that

cannot be accounted for in the products of the reaction (76).

Unfortunately, it is not clear how a reducing agent affects the enzymatic mechanism, but since tyrosinase is a cupro-enzyme, it is conceivable that the reductants are involved in the maintenance of an optimal cuprous:cupric ratio. It is also of considerable interest to note that tetrahydropteridine can replace ascorbate and DPNH *in vitro* (79).

In the presence of a suitable reductant, the unincorporated oxygen atom is reduced to water at the expense of two reducing equivalents, which can be provided for by any of the compounds referred to above. In the absence of such a reductant, dopa will serve this function, but in the process, dopaquinone is formed. As mentioned previously, there are conditions in the cell when dopaquinone formation is undesirable, namely when that dopa is needed for synthesis of sclerotin. It is conceivable that reducing agents such as ascorbate, DPNH and tetrahydropteridine can circumvent this occurrence *in vivo*.

The monophenol-diphenol oxidase system is extremely complex. It has been demonstrated by a number of investigators that the presence of both substrates (tyrosine and dopa) antagonize one another in a competitive manner (67,73). Hence, it is obvious that,under the simplest of reaction conditions, after a short period of time, a number of possibly competing reactions ensue, thereby obfuscating attempts to define stoi-

chiometry and reaction mechanism.

Insect tyrosinases. Although insect tyrosinase systems have yielded a paucity of information concerning the chemistry of the hydroxylation reaction, a great deal of information has been accrued which bears on the question of the involvement of tyrosinase in mediating the synthesis of dopa that is destined to participate in the sclerotization process.

As in the cases mentioned above, there exists a multiplicity of forms of tyrosinase within a given species of insects. Many studies have been concerned with eliciting the catalytic differences among the various forms observed. Ideally, one would like to ascribe functions to these distinct entities, that is, is there a specific tyrosinase responsible for the melanization pathway and another one capable of meeting the exigencies of the sclerotization pathway? It should be kept in mind that the sclerotization process may well require two distinct tyrosinases, one to synthesize dopa, the other to oxidize the dopa derivative to the reactive quinone.

Mills, et al. have shown in the cockroach system that the hemolymph tyrosinase is different from the one localized in the cuticle with respect to substrate specificities (80).

Similar results were obtained by Hackman and Goldberg in their studies on different species of Diptera (81). The cuticular enzyme displays good activity with dopamine and very little

with either tyrosine or dopa. A latent tyrosinase activity resides in the hemolymph, and, upon activation with sodium dodecyl sulfate, shows substrate specificity similar to that of the cuticular enzyme. However, a heat stable activator component isolated from the cuticle, when combined with the hemolymph pro-enzyme, extends the range of substrates utilized to include tyrosine and dopa. The latent hemolymph enzyme is observed at stages of development where cuticular tyrosinase activity is absent, the latter observed only just prior to puparium formation and immediately disappears as soon as the puparium is formed. These observations have limited physiological significance, for there is no evidence that the cuticular activator ever resides in the hemolymph, nor that the hemolymph pro-enzyme is an inactive form of that residing in the cuticle. If it were assumed that this were the case, means must be provided for the transport of the latent enzyme through the cuticle out to the epicuticle at the time of puparium formation. From Mitchell's work on Drosophila , there is no evidence for such a transport system (82).

Similar kinds of experiments in Calliphora have been described. A cuticular tyrosinase has been purified to crystallinity and it displays excellent activity with N-acetyl-dopamine and very little with monophenols (13). It will be recalled from the first section of the Introduction that N-acetyl-dopamine is the ultimate precursor to sclerotin for-

mation in this species, hence this cuticular enzyme seems to be unique in a functional context.

An active enzyme can be obtained by combining a latent activity found in the hemolymph with an activator isolated from the cuticle. If this active enzyme is then treated with mitochondria and centrifuged, it is observed that the supernatant contains only diphenol oxidase activity, while the fraction of activity that sediments with the mitochondria displays a high monophenol oxidase activity (83). These workers feel that the mitochondria provide a source of hydorgen donors which stimulates this activity. As in the previous case, the physiological significance of this effect is unclear.

A similar system has been described for the silkworm, Bombyx mori (84). Tyrosinase isolated from the hemolymph, in the presence of substrate, yields sigmoidal kinetics, implying that the system is undergoing a self-activation process. However, a heat labile activator isolated from the cuticle, upon addition to the hemolymph enzyme, results in a display of linear kinetics. A non-proteinaceous component may also be required. It is not known if the activator which may be present in the hemolymph is the same as the cuticular moiety, nor is it known if the hemolymph tyrosinase is the same as the one used in the cuticle.

The above examples indicate that there is a least a two-fold heterogeneity in tyrosinases, as defined by spatial dis-

criminations - one which resides in the hemolymph and the other in the cuticle. It is difficult to describe unequivocally the nature of the cuticular tyrosinase because of problems in isolation. Furthermore, constraints due to its orientation in the epicuticle may be placed on its in vivo activity. Also, as noted previously, the origin of the cuticular enzyme and its relationship to the one found in the hemolymph is dubious. It should be noted here that the hemolymph enzyme may be involved in wound healing (85).

Whitehead, et al. have reported the presence of an enzyme capable of oxidizing protocatechuic acid to a quinone in the egg capsule of Periplaneta (20). It will be recalled from the previous discussion on sclerotization that this compound is the reactive diphenol in sclerotization of the egg capsule. The enzyme does not oxidize tyrosine or dopa, prompting these investigators to term the enzyme a laccase instead of a tyrosinase. Little is known concerning the chemistry of this particular reaction.

Multiple forms of tyrosinase activity have been observed in extracts derived from Drosophila melanogaster larvae and pupae. The patterns observed are dependent upon genetic composition and stage of development (87). Furthermore, at least 5 distinct proteins (termed A_1,A_2,A_3,S and P) are necessary for full activation (88). All 3 A components, which are readily discernable upon polyacrylamide gel electrophor-

esis , give rise to active enzyme which supports the oxidation of dopa, however, only A_1 yields an enzyme which utilizes tyrosine as a substrate. This multiplicity of activities, as defined by sucrose gradient analysis, can be due to either different states of aggregation of the same active molecule or to discrete combinations of individual subunits, or to a combination of both of these factors. The different species of activity exhibit varying cresolase to catecholase ratios, a situation similar to that reported for the mushroom system (97,107).

The heterogeneity observed in tyrosinase populations can obviously be a reflection of unique activities associated with discrete forms of the enzyme. Tyrosinase may serve at least 4 distinct functions: 1.) hydroxylation of tyrosine to yield dopa, which then participates in the sclerotization process; 2.) oxidation of diphenolic derivatives to yield sclerotin; 3.) oxidation of tyrosine to yield dopa, which is then oxidized to melanin for pigmentation; and, 4.) oxidation of diphenols involved in wound healing. The only situation wherein one function has been ascribed to a unique species of tyrosinase is the enzyme isolated from Calliphora by Karlson and coworkers. This enzyme utilizes N-acetyl-dopamine as its sole substrate, hence this oxidase is likely responsible for catalysis of quinone formation in sclerotization. There are no other recorded cases which so strongly suggest a specific

function for a particular tyrosinase.

Hence, it is hazardous to conclude that tyrosinase is responsible for the hydroxylation of tyrosine in the sclerotization pathway *in vivo*. The fact that the monophenol and diphenol oxidase functions have never been physically separated from one another makes it appear unlikely that a tyrosinase, in the absence of a suitable regulatory system, is responsible for this hydroxylation reaction.

Tyrosine Hydroxylase

Tyrosine may also give rise to dopa via a reaction catalyzed by tyrosine hydroxylase. Although insect biochemists have paid scant attention to this possibility, this enzymatic system possesses features which make it an attractive candidate for the provision of dopa in the sclerotization pathway.

As in the case with phenylalanine hydroxylase, the most definitive enzymology on tyrosine hydroxylase has been done with mammalian material. In fact, the existence of tyrosine hydroxylase, per se, has not been described in insects. One possible explanation for this failure to detect the enzyme, if it does exist in insects, is that all insects have very active tyrosinase systems which can mask the hydroxylase activity.

The standard source of tyrosine hydroxylase material is

the bovine adrenal gland. The enzyme is particle bound and many of the early experiments were done with material that was gratuitously solubilized during the initial isolation procedures. However, the particulate and soluble enzymes are quite similar in their properties (89).

Tyrosine hydroxylase exhibits a requirement for tetrahydropteridine in much the same way as does phenylalanine hydroxylase (90). A number of likely reagents, such as, ascorbate, cysteine, ferrous chloride, flavin mononucleotide, flavin adenine dinucleotide and dopa, were tried as substitutes for pteridine and all were inactive. This is a critical piece of information, for ascorbate and dopa have very marked effects on tyrosinase. To substantiate further the distinction between the two enzymes, it was demonstrated that dopa is not a substrate for the hydroxylase, while, of course, it is an excellent one for tyrosinase (89). It was not recorded whether or not pteridine is capable of stimulating tyrosinase activity.

The stoichiometry of the tyrosine hydroxylase reaction is very much like that for phenylalanine hydroxylase (91):

$$\text{L-tyrosine} + DMPH_4 + O_2 \longrightarrow \text{L-dopa} + DMPH_2 + H_2O.$$

It had been reported that ferrous ion is a necessary constituent of this system and that a pre-incubation with this ion greatly stimulates the reaction (92). Shiman, et al.could not reproduce these effects using much purer material (91). Furthermore, they showed that ferrous ion can function to de-

stroy peroxide that is liberated during the non-enzymatic oxidation of the tetrahydropteridine. Peroxide is capable of deleterious interactions with the enzyme. It is possible, however, that ferrous ion is involved in the actual mechanism of enzyme action.

Dopa can give rise to norepinephrine via a decarboxylation to dopamine and a subsequent hydroxylation of the beta carbon of the side chain. It has been reported that norepinephrine is a competitive inhibitor of the pteridine (as was found in the phenylalanine hydroxylase system) (92). Dihydropteridine reductase, if present in sufficient quantities, antagonizes this inhibition by furnishing more reduced pteridine to compete with the catecholamine (93). These data have obvious significance in a regulatory context, exhibiting a classical end product inhibition phenomenon.

The most provocative result derived from the tyrosine hydroxylase system is that phenylalanine serves as a substrate for the enzyme (94). Phenylalanine is converted to tyrosine and, to a degree, to dopa. Since the enzyme preparations are fairly crude, one could argue that this activity is due to contamination with phenylalanine hydroxylase. However, if either α-methyl-tyrosine or 3-iodotyrosine is added and each reaction assayed, it is seen that both activities are inhibited to almost the same degree. Neither of these inhibitors affects purified rat liver phenylalanine hydroxylase activity,

and additionally, the liver enzyme does not hydroxylate tyrosine. The pH optima for the adrenal gland's capacity to hydroxylate phenylalanine and tyrosine are similar and differ from the liver's. These data suggest that the same enzyme localized in the bovine adrenal gland catalyzes the hydroxylation of both phenylalanine and tyrosine, and that it is distinct from the phenylalanine hydroxylase isolated from rat liver.

However, there are a few facts which appear difficult to reconcile with the one enzyme hypothesis. The Km for tyrosine is much lower than for phenylalanine, and, also, the Vmax for tyrosine is much higher than for phenylalanine. It is peculiar that in the case where phenylalanine is the substrate that tyrosine would ever accumulate,which it does. Ikeda, et al. conjecture that the enzyme - tyrosine complex must first dissociate before resumption of oxidation of tyrosine (94). Since phenylalanine is a competitive inhibitor of tyrosine in their material, it seems that this fact may account for the accumulation of tyrosine. Recently, Shiman, et al. , using a much more homogeneous enzyme preparation, demonstrated that, when the natural cofactor is used (the other experiments utilized the synthetic 6,7-dimethyl compound), the rate of conversion of phenylalanine to tyrosine is in the same range as that for tyrosine to dopa (91).

In summary, the enzyme isolated from mammalian adrenal glands behaves very much like mammalian phenylalanine hy-

droxylase, especially with regard to the tetrahydropteridine requirement. This enzyme is distinct from the classical tyrosinase in that the latter does not require pteridine for full activity and, also, it utilizes dopa as a substrate, whereas, tyrosine hydroxylase does not. More detailed and thorough investigations concerning this distinction have not been reported.

It is obvious that the existence of such a tyrosine hydroxylase system in insects would circumvent the problem that tyrosinase appears to possess in supplying dopa for sclerotization, namely the rapid subsequent oxidation of dopa to melanin. Since this enzyme does not appear to oxidize dopa any further, this metabolite would then be readily available to participate in sclerotin formation.

MATERIALS AND METHODS

1. Drosophila culture

All stocks used in these studies were obtained from the stock supply maintained at Caltech. Egg collection and animal rearing techniques were carried out according to Mitchell and Mitchell (101). In most cases, early to middle third instar larvae were used as the source for phenylalanine hydroxylase and late third instar for the enzymes involved in the hydroxylation of tyrosine. Since early and middle third instar larvae are still feeding, a method had to be devised to obtain larvae free from the yeast and cornmeal. This separation may be readily accomplished by suspending the food-larvae mixture in 10% sodium chloride (97). The larvae float on top while the food settles to the bottom. This process is repeated one time, followed by extensive rinsing with distilled water. The larvae are allowed to air dry at room temperature and then frozen in liquid nitrogen and stored at -80°C. Samples can be stored at this temperature for as long as 6 months without any noticeable effect on the enzymes studied.

In most studies, Oregon R (wild type) stocks were used, however, in a few cases, ebony was employed. There appears to be no difference between these two strains with respect

to the enzymes studied. The same strain was maintained throughout a single series of experiments.

2. Injection of substrate into larvae and subsequent processing

Uniformly labelled C^{14}-phenylalanine was injected into third instar larvae using a finely drawn glass needle. Approximately 0.05µl (around 5.5×10^5dpm) were administered per animal. These injections were performed by Dr. H. K. Mitchell, to whom I am indebted for this service. After an incubation period of 10-15 minutes, the animals were washed from the microscope slide to which they were attached by "double-stick" tape and then squashed with a stirring bar on a piece of Whatman 3 MM chromatography paper. The paper was subjected to descending chromatography in n-butanol/acetic acid/water (4:1:5) overnight. The papers were dried and then cut into strips (1 x 3 centimeters), each individual strip placed in a scintillation vial along with 10 ml. of scintillation fluid (4g PPO + 50mg POPOP per liter of toluene) and counted in a Beckman LS-200B scintillation counter. Each sample was usually counted to a pre-set error of 5%. C^{12}-tyrosine standards were run along and tyrosine was detected by its reaction with ninhydrin (0.5% in acetone). In all cases, a peak of radioactivity was seen in chromatograms corresponding to the ninhydrin spot.

3. Preparation of extracts

It was found that the most reliable method for obtaining extracts involved first grinding the frozen larvae in a mortar and pestle which had been pre-chilled in liquid nitrogen along with a quantity of sand equivalent to one half the weight of the larval sample. The finely ground powder resulting from this procedure was then extracted in buffer for a few minutes, usually until all of the powder had thawed. The extraction volume to sample weight ratio was usually kept at 10. It was necessary to include in the extraction buffer a reagent capable of arresting phenol oxidase activity. Dithiothreitol (DTT) at a concentration of 1-2mM served this purpose. The extraction liquid was either 0.1M phosphate or 0.1M Tris maintained around pH 7 when phenylalanine hydroxylase was to be assayed and 0.1M phosphate, buffered at pH 6.1 when "tyrosine hydroxylase" was assayed. Both extraction buffers contained DTT. No darkening of the extracts, indicative of phenol oxidase activity, was ever noted under these conditions. Hereafter, all procedures were carried out at 4°C. The extract was filtered through a double layer of gauze to remove lipid and was then centrifuged in a refrigerated Servall at 10,000 r.p.m. for 15 minutes, with the supernatant being

used for subsequent experiments in all cases. An aliquot of the supernatant was passed over a Sephadex G-25 column at a bed volume to sample volume of 8. In the phenylalanine hydroxylase situation, the elution buffer was the same as the extraction buffer, whereas when the preparation was to be used in "tyrosine hydroxylase" assays, DTT was deleted from the elution buffer.

4. Assay for phenylalanine hydroxylase

The usual assay mixture contained the following components: 0.4μmoles of phenylalanine. 0.064μmoles $DMPH_4$, 0.1μmoles DTT and either 10μmoles potassium phosphate or Tris, the final pH of the mixture maintained close to 7. The total volume was 0.2ml. Major deviations from this scheme in individual experiments are pointed out when necessary. $DMPH_4$ was made up in 5mM HCl and aliquots were stored at -20°C for months without any loss of cofactor activity. The enzyme preparation was the penultimate addition to the assay and the reaction was initiated upon the addition of $DMPH_4$. The reaction was allowed to proceed for 25 minutes at room temperature in open tubes. When the amount of tyrosine synthesized was to be determined by the fluorescense technique (see below) the reaction was stopped by the addition of 0.2ml ice cold 15% trichloroacetic acid (TCA), and in the case of the paper chromatography assay for newly

synthesized tyrosine, the tubes were merely immersed in a water bath held at 75°C for a few minutes. If the concentration of protein in the assay was too small to give a flocculent precipitate upon addition of TCA, 50µl of bovine serum albumin at 3 mg/ml were added to insure that all of the protein was removed.

A. Assay for tyrosine by the fluorescence method.

A number of slight variations on this procedure were used during the course of these studies, but I shall only describe the one most commonly used. Suffice it to say, that whenever the volumes or concentrations were perturbed, new standard curves were derived to allow for these changes.

Basically the technique is that described by Waalkes and Udenfriend (98) with variations as suggested by Geiger (103). The tubes resulting from the enzyme assay described above were de-proteinized by centrifugation at 8,000 r.p.m. for 10 minutes at 4°C. The supernatant was carefully drawn off with a Pasteur pipet and placed in a tube fitted with a ground glass stopper. The tubes may be stored at -20°C at this point for future assay without any abnormalities arising in subsequent readings. 0.8 ml of nitroso-napthol "reagent" were added to the 0.4 ml protein-free supernatant and allowed to react at 55°C for 30 minutes. The tubes were lightly shaken during this period. The composition of the nitroso-napthol "reagent" was as follows (and the order of addition

of the various components): 2 parts of 3N HNO_3, 2 parts of 2.5% sodium nitrite, 1.3 parts of 0.5 mg/ml (in 95% ethanol) nitroso-napthol and 1 part of 95% ethanol. After the 30 minute incubation, the tubes were allowed to cool to room temperature and then 10 ml of 25% ethanol were added to each tube. Either a Farrand Model MK-1 or a Hitachi Perkin-Elmer Model MPF-2A spectrofluorometer was used to measure the fluorescence. The samples were excited at 465 mμ and the fluorescence at 565 mμ was measured. It has been reported that tyramine will also give this reaction, however, it was not tested in this system. Phenylalanine did not react under these conditions.

A non-substrate or cofactor control was always included for each determination to blank out the tyrosine that is not derived via the phenylalanine hydroxylase catalyzed reaction. When low levels of activity were measured, a control for non-enzymatic hydroxylation was also included.

Although this method is relatively sensitive and simple, it was not without a few objectionable features. The readings were not always readily reproducible, even for the same samples. Replicate determinations were usually necessary and the same samples were read many times in any attempt to achieve a level of consistency.

B. Assay for tyrosine by the paper chromatography technique

The concentrations of the components in the enzyme reac-

tion where the paper chromatography assay for tyrosine was to be used were essentially the same as when the fluorescence assay was utilized. The only difference being that a fraction of the phenylalanine was uniformly labelled with C^{14}. Approximately 1μc of label was included per assay tube. Either a pre-heated enzyme preparation as a substitute for an active preparation or a tube lacking $DMPH_4$ was used as a control to blank out tyrosine present as a contaminant in the commercial isotope sample. Once the reaction was terminated by the heat treatment, a 25μl aliquot from each tube was streaked onto a pre-cut piece of Whatman 3 MM paper (3 x 40 centimeters). The streaks were allowed to dry and then subjected to descending chromatography and subsequent analysis as described in section 2.

This technique is very reliable in comparison with the previously described fluorescence assay. Also the need for a non-substrate or non-cofactor blank for each determination is not necessary in this system, since all of the tyrosine scored arises from phenylalanine. This is also a more sensitive assay and can be used to measure very low levels of activity. One does not have to be concerned with the fluorescence of endogenous material which could be a source of some of the fluctuations encountered in the fluorescence assay. The major drawback to this assay is that it is both time

consuming and expensive.

5. Assay for the enzyme(s) responsible for the hydroxylation of tyrosine

The basic assay mixture contained the following components in a total volume of 200 µl: 15-20µ moles of potassium phosphate, enzyme usually in a volume of 25µl, tyrosine anywhere from 0.02-0.08µ moles (including approximately 10^6 dpm of uniformly labelled C^{14}-tyrosine), and $DMPH_4$ ranging from 0.02-1.6µ moles. The exact concentrations of the latter two compounds are given for each individual experiment. Oftentimes, the enzyme was diluted 2-4 fold prior to use in the assay. When necessary, 0.1-0.2µ moles of DTT was present. The pH of the assay was maintained at 6.1. $DMPH_4$ was usually made up at a concentration of 16 mM in 0.1M potassium phosphate buffered at pH 6.7. The resulting pH of this solution is 6.1. Appropriate dilutions of $DMPH_4$ were made in pH 6.1 buffer.

In general, $DMPH_4$ was the penultimate addition and the reaction was initiated upon addition of the enzyme. The reactions were allowed to proceed in capped tubes protected from light (which inactivates the pteridine) for anywhere from 25-40 minutes at room temperature, whereupon the tubes were immersed in a water bath maintained at 75°C to stop the

reaction. After cooling in ice, 25µl aliquots were streaked onto pre-cut strips of Whatman 3 MM chromatography paper (3 x 40 centimeters) and the strips were subjected to descending chromatography in a mixture of n-butanol/acetic acid/water (4:1:5) overnight. The developed chromatograms were then treated in the same manner as described in section 2. An authentic dopa standard was run in each experiment and its position marked on the strip by its reaction with ninhydrin.

In most assays, a reaction was carried out in the absence of enzyme to derive a value for non-enzymatic hydroxylation of tyrosine to dopa. Appropriate subtractions were made to yield a value corresponding to the level of product that arises purely enzymatically.

As will be presented in the Results section, two products of further dopa metabolism appear in the chromatograms, namely the material at the origin, which represents melanin or its precursors and peak I.

This technique proved to be reasonably reliable and it has the additional advantage of revealing the other two synthetic activities. However, the procedure is extremely time consuming.

6. Phenol oxidase assay

Phenol oxidase (tyrosinase) was routinely assayed for by the increase in optical density at 475 mµ, the wavelength at

which dopachrome (an oxidation product of dopa and precursor to melanin) maximally absorbs under conditions described by Mitchell (105). Readings were taken on a Cary 15 spectrophotometer with a one centimeter cell.

Either tyrosine or dopa at a concentration of 2 mM was used as substrate and note will be made which is used in individual experiments.

7. Ammonium sulfate fractionation.

Two fractions of protein were derived from larval extracts in these studies: material insoluble in the 0-40% saturation range and material insoluble in the 40-60% range.

Saturated ammonium sulfate (made up in 0.1M phosphate, pH 7.0 without readjustment of the pH) was slowly dripped into a 10:1 (v/w) larval extract to a final level of 40% saturation. The solution was stirred for 15 minutes and was then centrifuged either at 25,000 r.p.m. for 10 minutes in a type 30 rotor in a Beckman model L or at 9000 r.p.m. for 30 minutes in a GSA rotor in a Servall. The supernatant was brought to 60% saturation by slowly adding in more salt. Again the mixture was allowed to stir for 15 minutes and was centrifuged in the same manner as above. The pellet resulting from this centrifugation was washed occasionally in 60% ammonium sulfate (in approximately 8% of the extraction volume) and centrifuged at 20,000 r.p.m. for 5

minutes in a type 40 rotor in a Beckman Model L ultracentrifuge. When these preparations were to be used in phenylalanine hydroxylase experiments, the pellets were resuspended in approximately 5% of the original extraction volume in either phosphate or Tris buffer at neutral pH's containing 1 mM DTT. In the case where "tyrosine hydroxylase" was to be assayed, the pellet was taken up in the same volume but in a phosphate buffer maintained at pH 6.1 with no DTT present. The suspended material in all cases was then centrifuged at 20,000 r.p.m. for 5 minutes to remove the insoluble material. The supernatant was then passed over a Sephadex G-25 column as described in section 3 and stored at -80°C. The supernatant from the last centrifugation step need not be treated with G-25 immediately, but can be stored at -80°C and used at a later date.

8. Gel filtration.

Gel materials were swollen in elution buffer for times prescribed by the manufacturers (Pharmacia and Bio-Rad). Fines were removed by repeated suspensions and decantations. The gels were stored at 4°C, usually under butyl alcohol to prevent microbial contamination. Columns were run under pressure heads prescribed by the manufacturers for each type of gel.

9. Sucrose gradient centrifugation

Sedimentation equilibrium gradients in sucrose were employed to yield relatively pure preparations of phenol oxidase. The procedure used was essentially that described by Mitchell et al. (87). 30 milliliter gradients run for 16 hours at 14,000 r.p.m. in a Beckman type 25.1 rotor at 4°C were utilized.

"Tyrosine hydroxylase" activity of each fraction was assayed for by the paper chromatography technique (see section 5), and phenol oxidase activity was determined by the assay for dopachrome formation (see section 6) using dopa as the sole substrate.

10. Protein determinations

Protein was assayed for either by its absorbance at 280 mμ or by the Lowry technique (104). Bovine serum albumin was used to derive a standard curve for the latter method.

11. Reagents

Phenylalanine, tyrosine (both A grade), dithiothreitol (A grade),6,7-dimethyl-5,6,7,8-tetrahydropteridine (B grade), TPNH (A grade) and NADH (A grade) were all purchased from Calbiochem. 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine was obtained from Aldrich. (D,L)-C^{12}-dopa and L(+)-

ascorbic acid came from Sigma. Nitroso-napthol (melting point = 107-109°C) was a product of Matheson Coleman Bell. NSD-1015 is a product of Sandev, Ltd. Mushroom tyrosinase was obtained from Worthington.

Uniformly labelled C^{14}-phenylalanine and tyrosine, both at specific activities = 450-460 mc/mmole, were purchased from either Schwarz-Mann or New England Nuclear. In the latter case, the material is stored in 1.ON HCl and neutralization with 1 N NaOH was carried out prior to use. C^{14}-dopa, labelled in the 3 position, was acquired from Amersham-Searle in the solid state (specific activity = 21 mc/mmole). Solubilization was achieved with 1 ml. of 0.01 N acetic acid.

Scintillation fluid was derived from Liquifluor (New England Nuclear) diluted with reagent grade toluene.

All other chemicals used were of reagent grade quality.

RESULTS

1. Conversion of Phenylalanine to Tyrosine

Detection of in vivo activity responsible for the conversion of phenylalanine to tyrosine

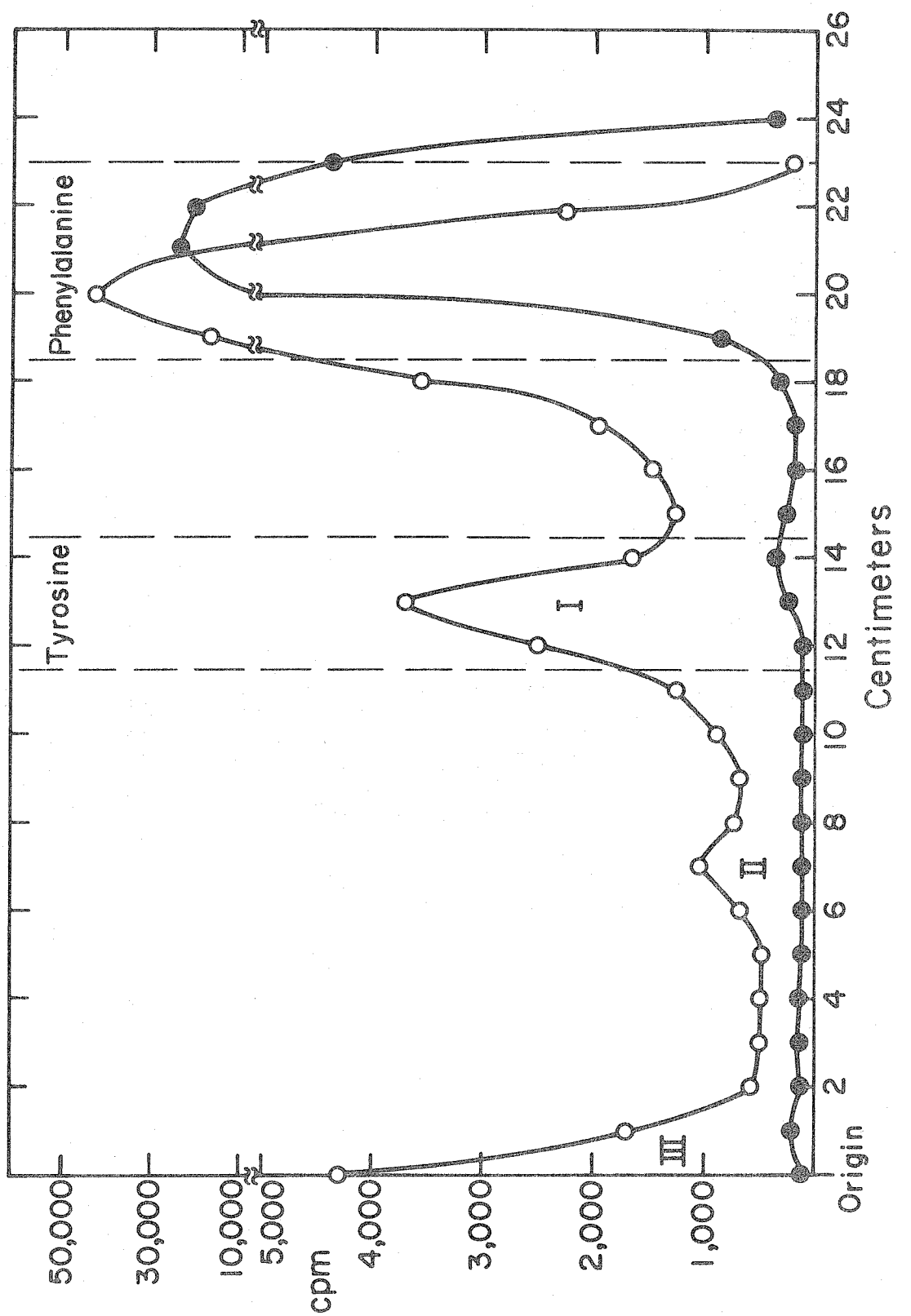
As was pointed out in the Introduction, conversion of phenylalanine to tyrosine in silkworms was demonstrated by injection of C^{14}-phenylalanine into larvae and tyrosine was subsequently detected in the silky cocoon fibers.

A similar type of experiment was employed to measure Drosophila larvae's ability to catalyze this conversion and the results are depicted in Figure 1. Three new peaks of radioactivity arise as a result of the in vivo incubation. It should be noted that there are no appreciable levels of synthetic activity reflected in the region to the right of phenylalanine, so that this part of the chromatogram is deleted in the figure. C^{12}-tyrosine was run along as a standard and its migration, as evidenced by the ninhydrin reaction, corresponds to peak 1. From subsequent experiments to be described in the second chapter of the Results, peak 2 represents dopa, while peak 3, or the origin material, represents oxidation of dopa to melanin.

The same sort of injection experiments were carried out and analyzed in three different chromatographic systems: n butanol/acetic acid/water (4:1:5), phenol/n butanol/water/

Figure 1. Detection of an *in vivo* activity capable of converting phenylalanine to tyrosine. Three wild type (Oregon R) third instar larvae were injected with uniformally labelled C^{14}-phenylalanine and processed as described in Materials and Methods, section 2. Included is a depiction of the chromatography of an untreated sample of C^{14}-phenylalanine (closed circles).

The R_f values for phenylalanine and tyrosine varied a slight bit among the chromatograms, a situation which is reflected in this figure.


Phenylalanine
Tyrosine
I
II
III
Origin
Centimeters
cpm
50,000
30,000
10,000
5,000
4,000
3,000
2,000
1,000
2
4
6
8
10
12
14
16
18
20
22
24
26

acetic acid (25:12.5:25:5) and phenol/citric acid (5% citrate in 50% aqueous phenol). In each case, a peak of activity was found corresponding to the tyrosine region of the particular solvent system. The dopa peak was not always observed, the probable reason being that dopa synthesis is minimal during some of the stages of development used in these experiments. The best separation of tyrosine from unreacted phenylalanine was achieved by the butanol/acetic acid/water system, which was used for all subsequent chromatography assays.

The percentage of conversion of phenylalanine to tyrosine can be derived from these chromatograms, with account being taken of peaks 2 and 3, which, as will be seen in the second section of the Results, are derived from tyrosine. Three experiments of this type yielded values of 16-20%, while one experiment gave a value of 40%.

An experiment was performed to ascertain the animal's total capacity to carry out this conversion. Increasing amounts of phenylalanine were injected into larvae and the same analytical procedures were followed as described above. There was no hint of saturation of the larvae's capacity to hydroxylate tyrosine. The highest concentration of phenylalanine injected was 0.4 nanomoles per larvae.

Screening of mutants for phenylalanine hydroxylase activity

A number of mutants were investigated for their capacities to convert phenylalanine to tyrosine. It was thought that body color and eye color mutants would be likely candidates for demonstrating abnormal metabolism of phenylalanine. A defect in the animal's ability to synthesize tyrosine may be reflected in its coloration, since tyrosine participates in sclerotization and melanization, two processes responsible for pigmentation. Eye color mutants were also thought to be of interest, for the material responsible for eye pigmentation is derived from an essential cofactor of phenylalanine hydroxylase, the pteridines.

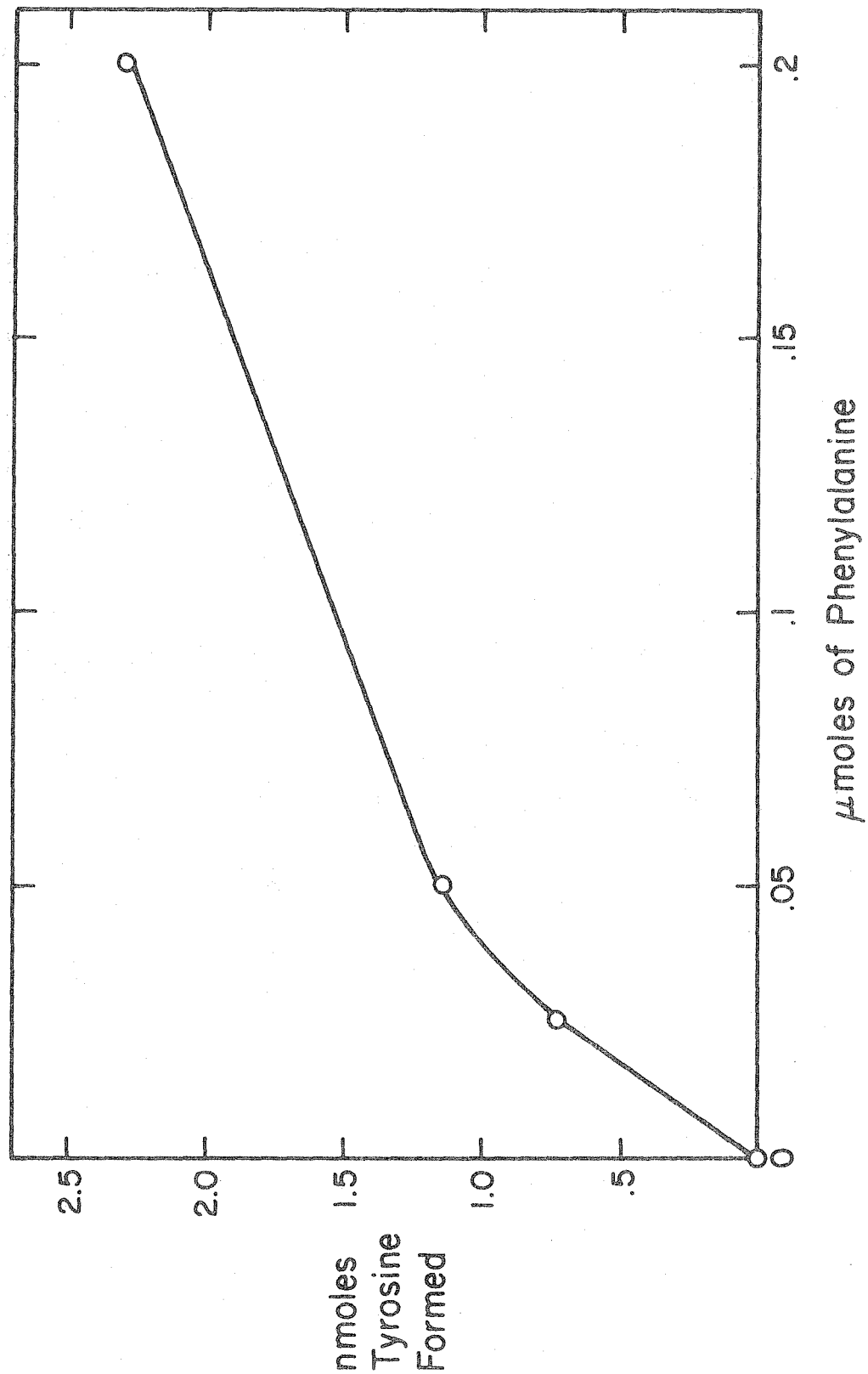
Larvae from ebony (e), a body color mutant, and the following eye color mutants: brown (bw), white (w), purple (pr), and maroon-like (mal) were tested for their abilities to convert phenylalanine to tyrosine by the injection method described above. No substantial deviation from wild type activity was seen in any of the strains tested. Slight differences were noted, but they could have been due merely to discrepancies in the ages of the animals used. To explore fully these possibilities, different stages of development should be analyzed for each mutant and appropriate comparisons made.

Detection of an in vitro activity capable of converting phenylalanine to tyrosine

A crude larval extract was assayed for its ability to support the conversion of phenylalanine to tyrosine. The results from such an experiment are described in Figure 2. The crude extract was quite capable of catalyzing this conversion. The system began to saturate at a final phenylalanine concentration of 0.33 mM. This number is actually too low since the endogenous phenylalanine has not been taken into account.

If it is assumed that the average weight of each larvae used in this experiment is 2 milligrams (95), the highest level of activity (2 nanomoles of tyrosine synthesized/100µl of extract) calculates out to approximately 0.4 nanomoles of tyrosine synthesized/larvae. This compares to a figure of 0.12 nanomoles of tyrosine synthesized/larvae from the in vivo injection experiments. As was noted, however, there was no hint of saturation of this system, therefore, it is not possible to compare the two systems by this method. If one looks at the percentage of conversion in the linear phase of the activity response to substrate concentration (nanomoles tyrosine formed/nanomole phenylalanine input), the in vivo figure is approximately one order of magnitude greater than is the in vitro's. It should be noted here that yeast, which

Figure 2. The ability of a crude extract to convert phenylalanine to tyrosine. Various concentrations of phenylalanine were added to a fixed amount of extract (approximately 1mg protein/ 100 µl) and allowed to react for 25 minutes. In this instance, the entire larval extract was used - there was no centrifugation or treatment with Sephadex G-25. The extract was made at a ratio of 10:1 (v/w). Tyrosine was assayed by the paper chromatography technique (see Materials and Methods, section 4B).


nmoles Tyrosine Formed
2.5
2.0
1.5
1.0
.5
0
.05
.1
.15
.2
μmoles of Phenylalanine

the animals feed upon and could be a possible source of contamination, are completely incapable of converting phenylalanine to tyrosine in the standard assay (Materials and Methods, section 4).

Validity of the fluorescence assay

Before any further discussion on the nature of the hydroxylase system, a description of the second method of assaying for tyrosine will be presented. This method takes advantage of the fluorescence characteristics of a tyrosine: nitroso-napthol derivative. The exact chemical nature of this substance is unknown, however, when excited with light of wavelength of 465 mμ, it gives an emission spectrum whose maximum is at 565 mμ. The reaction conditions are described in Material and Methods, section 4A.

A standard curve for the assay is depicted in Figure 3. The curve is linear throughout a wide range of tyrosine concentrations, so that it was a fairly simple matter to derive samples with appropriate concentrations of tyrosine for the assay. It was sometimes necessary to dilute first an aliquot from the enzyme assay to fit it into this range. The dilutant was made up of the same proportions of components present in the aliquot. Occasionally, internal standards were run to verify that the fluorescence recorded was, in fact, due to the tyrosine: nitroso-napthol derivative.

Figure 3. Standard curve for the fluorescence assay for tyrosine. Varying concentrations of tyrosine were added to the usual components of the enzyme assay system (see Materials and Methods, section 4), including TCA and excluding protein, which was replaced by an equal volume of buffer. The nitroso-napthol reaction with tyrosine was carried out as described in Materials and Methods, section 4A. The ordinate represents fluorescence readings at 565 mμ when the samples are excited with light of wavelenth 465 mμ. A reagent blank (minus tyrosine) was subtracted from each value.

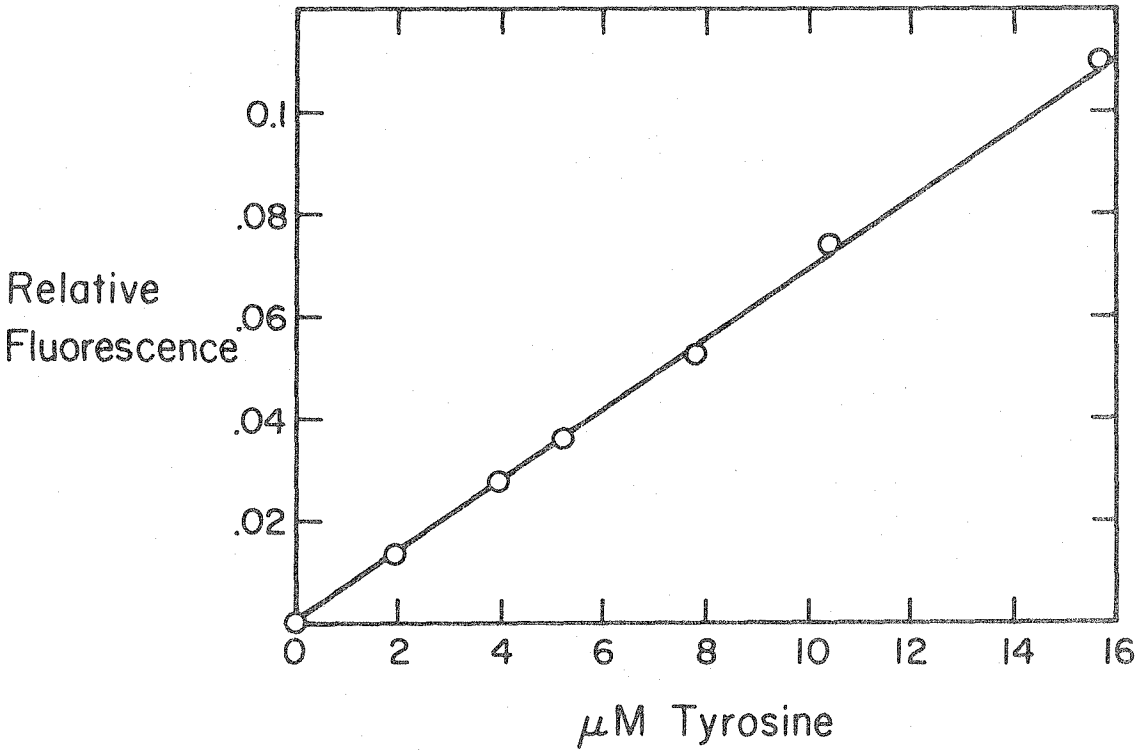
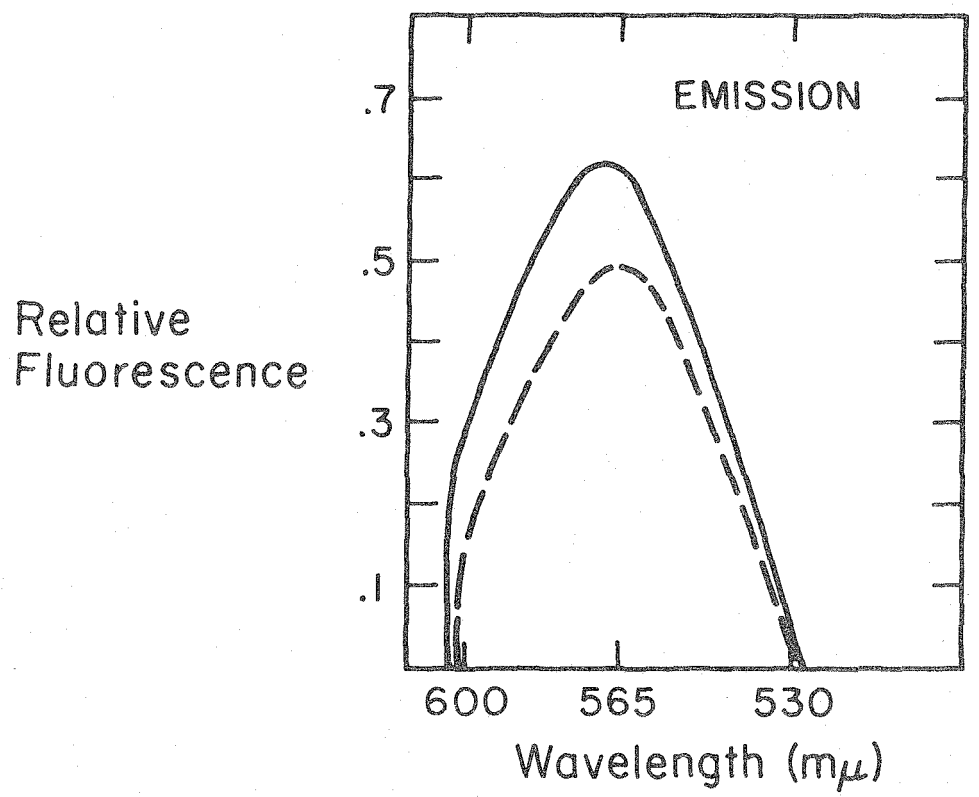
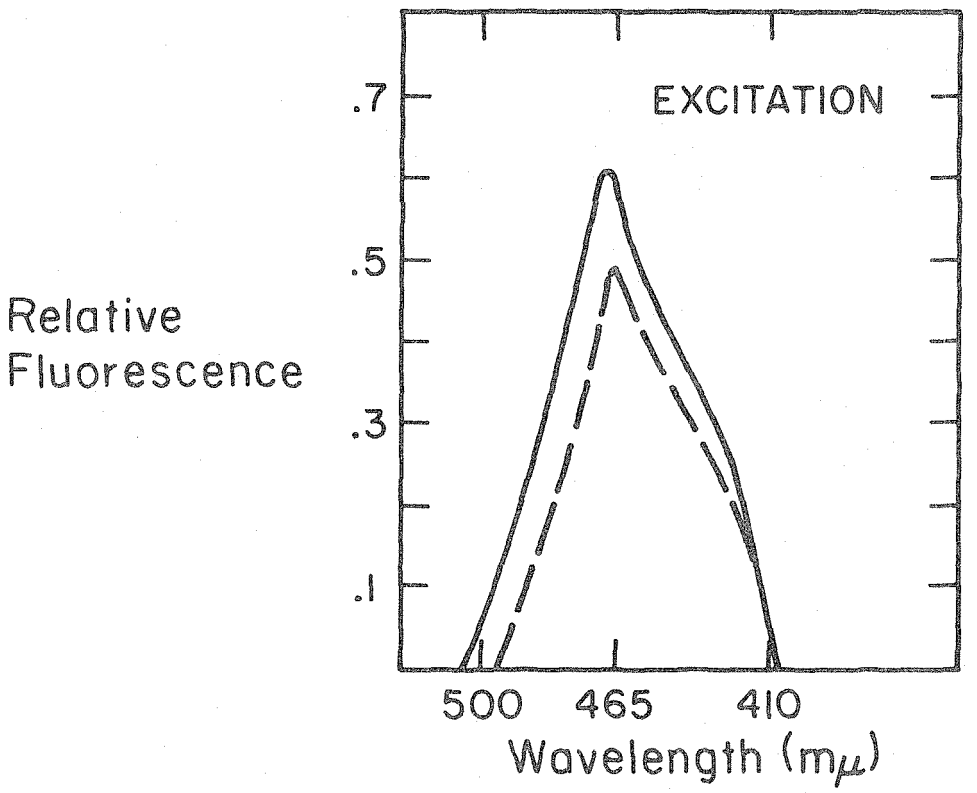

Relative
Fluorescence
0.1
.08
.06
.04
.02
0
2
4
6
8
10
12
14
16
μM Tyrosine

Figure 4. Excitation and emission spectra of the nitroso-napthol:tyrosine derivative. The top graphs depict emission spectra (excitation at 465 mμ) for authentic tyrosine (solid line) at a final concentration of 8 μM and the material derived from an enzyme assay (dotted line). The nitroso-napthol assay was performed as delineated in Materials and Methods, section 4A.

The bottom graphs represent the corresponding excitation spectra (emission at 565 mμ). The notations are the same as above. These readings were taken on a Farrand Model MK-1 spectrofluorometer.


EMISSION
Relative Fluorescence
.7
.5
.3
.1
600
565
530
Wavelength (mμ)


EXCITATION
Relative Fluorescence
.7
.5
.3
.1
500
465
410
Wavelength (mμ)

To substantiate further the validity of the assay, excitation and emission spectra were derived for samples of authentic tyrosine and the material derived from enzyme assays (see Figure 4). The spectra are identical, indicating that it is tyrosine that is being measured in the assay.

The original protocol for this assay called for an extraction of the unreacted nitroso-napthol with 1,2-dichloroethane. It was found that this step was unnecessary if the sample were merely diluted with 25% ethanol.

Delineation of the components necessary for the conversion of phenylalanine to tyrosine

Natural products When a soluble larval extract was treated with Sephadex G-25 and the excluded material assayed for hydroxylase activity, none was observed. If the included material from the G-25 column was added back, partial restoration of activity occured, as is seen in Table 1. The reconstitution achieved (line 3) is a minimal figure because the included material was extensively diluted and not reconcentrated prior to assay. Furthermore, the included material also contains substrate, so one is effectively decreasing the specific activity of the radioactive phenylalanine in the assay. This type of experiment was repeated many times with varying results. The reconstitution observed at times could possibly be accounted for by non-enzymatic hydroxylation of phenylala-

Table 1

The Necessity for a Low Molecular Weight Compound in the Conversion of Phenylalanine to Tyrosine

components present	cpm in tyrosine region*
200 µl untreated extract	5250
200 µl excluded on G-25**	400
100 µl excluded + 100 µl included	1000
200 µl included	400
100 µl heated excluded + 100 µl included	400

* 3×10^5 dpm of C^{14}-phenylalanine was incubated with the enzyme and G-25 included preparations. Tyrosine was assayed by the paper chromatography technique (see Materials Methods, section 4B).

** Extracts were treated with Sephadex G-25 (Materials and Methods, section 3). After the void volume (excluded material) was collected, the included fraction was eluted with a few column volumes of buffer.

nine.

Synthetic cofactors It was stated in the Introduction that a tetrahydropteridine is a necessary cofactor for phenylalanine hydroxylase activity. 6,7-Dimethyl-4-amino-tetrahydropteridine ($DMPH_4$), a synthetic pteridine, when added to the excluded fraction from a Sephadex G-25 column, yielded very good activity, as can be seen in Table 2. When the dilution of the enzyme is considered, the activity with the synthetic cofactor is in the same range as the unfractionated system.

Even though activity could be achieved with natural factors (G-25 included material), it was thought best to utilize synthetic pteridines because of the crudity of the natural system. Kaufman's work on the enzyme suggested that, if pteridines were used in great enough excess, there would not be a need for any further components, that is, a TPNH linked pteridine regeneration system (34). Moreover, Bublitz has demonstrated that DTT is capable of converting the oxidized pteridine back to the reduced state (114).

The effect of other reductants on hydroxylase activity

A number of biological reductants were assayed for their ability to replace pteridine in the hydroxylase system. Tetrahydrofolate, NADH, TPNH and ascorbic acid were tested and none displayed any ability to stimulate the reaction.

Table 2

The Ability of a Synthetic Pteridine to Support the Conversion of Phenylalanine to Tyrosine

components present	cpm in tyrosine region*
200 µl untreated extract	12,000
200 µl excluded on G-25	3,000
100 µl excluded + 100 µl 0.64 mM $DMPH_4$	7,000
100 µl heated excluded + 100 µl 0.64 mM $DMPH_4$	3,000

* The conditions for this assay were similar to those described for Table 1, except that the synthetic pteridine is used in lieu of G-25 included material.

A pteridine containing reaction was performed in each assay to establish the enzyme's capacity to be stimulated by a cofactor.

A number of experiments were performed in the presence of both pteridine and TPNH to determine if the latter compound might be involved in regenerating the former and consequently stimulating the synthesis of tyrosine. The results from these experiments were varied; at times, a distinct enhancement by TPNH (in the presence of $DMPH_4$) was noted, while at other times, no effect was seen. However, it was clear that DTT (in the presence of $DMPH_4$) was more effective in stimulating the reaction than was TPNH even when this factor did display enhancement abilities. It is also possible that DTT stimulates the system in other ways than merely allowing for efficient reduction of the dihydropteridine. Hence, DTT was always present to aid in maintaining optimal reaction conditions.

Characteristics of Drosophila phenylalanine hydroxylase

This section deals with the enzymological characteristics of phenylalanine hydroxylase in the relatively crude state. The enzyme used in these studies was derived from one of two sources: either a crude extract that has been centrifuged at 10,000 r.p.m. and desalted over Sephadex G-25 (Materials and Methods, section 3), or a 40-60% ammonium sulfate fraction (see Materials and Methods, section 7).

Kinetics

A time course of the phenylalanine hydroxylase catalyzed reaction is depicted in Figure 5. There is no indication of a lag period before onset of tyrosine accumulation. The reaction proceeds in a linear fashion for twenty minutes, whereupon it essentially ceases. It is not clear why the rate should decline so rapidly. It is most likely due to comsumption of $DMPH_4$, although DTT was present in this experiment to assist in the recycling process.

Included along with the complete system is a depiction of the kinetics of substrate-independent accumulation of tyrosine. This activity is likely due to a proteolytic enzyme.

Amount of tyrosine synthesized as a function of enzyme concentration

Figure 6 demonstrates the accrual of tyrosine with varying amounts of enzyme present. There is no indication that the hydroxylase must undergo any sort of homotropic interactions to achieve maximal activity. It should be noted that oftentimes in a experiment of this nature, a significant amount of tyrosine can be synthesized non-enzymatically, as has been described elsewhere (109). This fact must be

Figure 5. Kinetics of the phenylalanine hydroxylase catalyzed reaction. The enzyme preparation used was the 40-60% ammonium sulfate fraction (see Materials and Methods, section 7). Two reaction tubes were set-up:one with phenylalanine, the other without it. At various times, 0.2ml aliquots were withdrawn and tyrosine assayed by the fluorescence method (see Materials and Methods, section 4A). To obtain the values for hydroxylase derived tyrosine, the amount of tyrosine generated in the absence of phenylalanine is subtracted from that amount accumulated in the presence of phenylalanine.

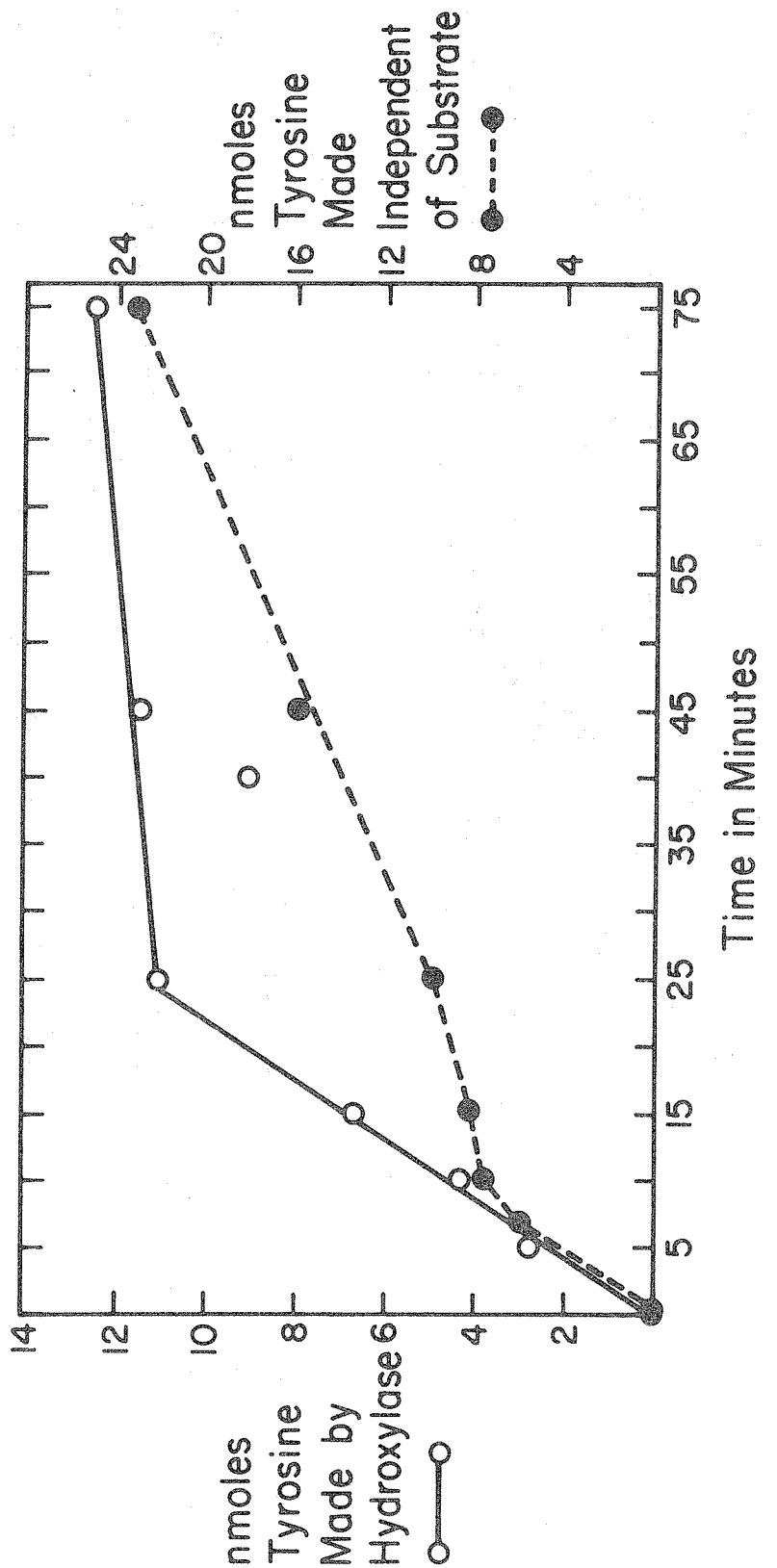
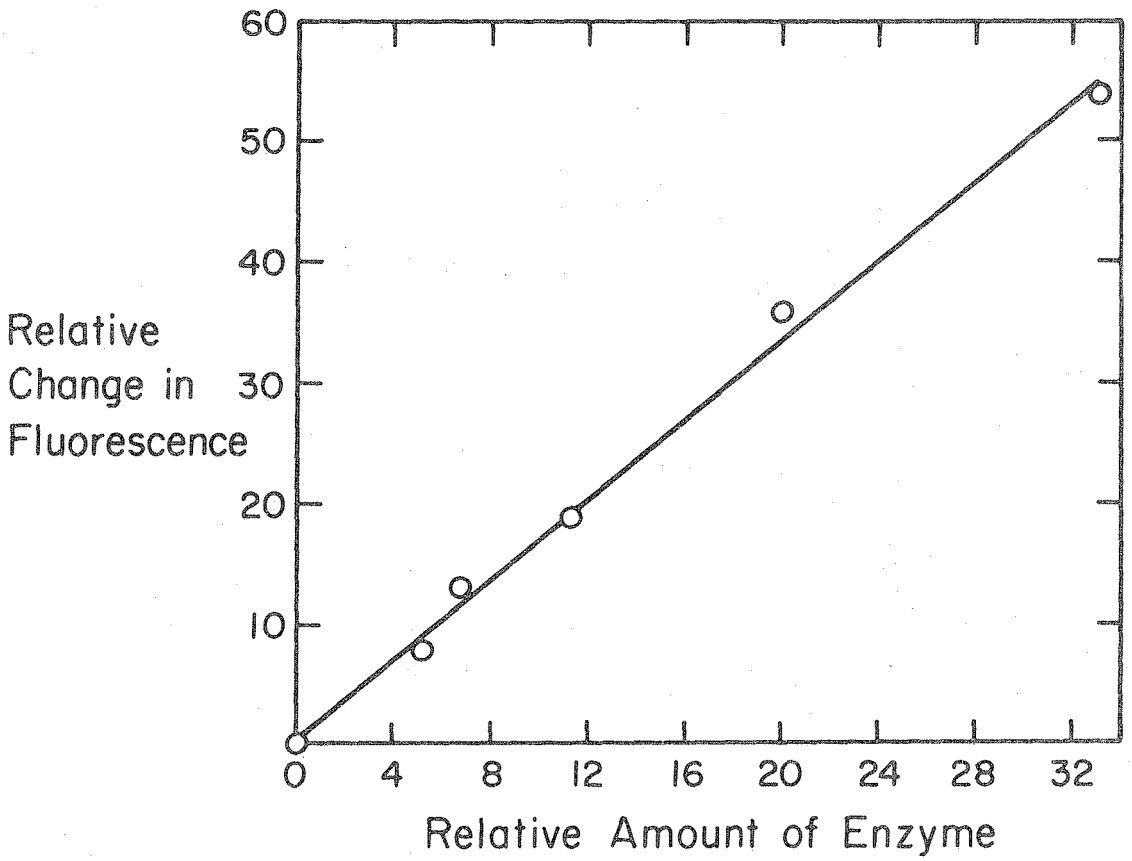

nmoles Tyrosine Made by Hydroxylase
14
12
10
8
6
4
2
nmoles Tyrosine Made Independent of Substrate
24
20
16
12
8
4
5
15
25
35
45
55
65
75
Time in Minutes

Figure 6. The synthesis of tyrosine as a function of hydroxylase concentration. Varying amounts of enzyme (40-60% fraction-see Materials and Methods, section 7) were included in the standard assay system (see Materials and Methods, section 4). The fluorescence assay was used to detect tyrosine (see Materials and Methods, section 4A). The ordinate values are a reflection of phenylalanine hydroxylase activity. Each value is derived by subtracting substrate independent tyrosine values from the overall reaction values.


60
50
40
30
20
10
Relative
Change in
Fluorescence
0
4
8
12
16
20
24
28
32
Relative Amount of Enzyme

taken into account when working with small quantities of the enzyme.

Phenylalanine hydroxylase activity as a function of phenylalanine concentration Phenylalanine hydroxylase displays a normal saturation curve with respect to its substrate, as summarized in Figure 7. Although the system is saturated at a final substrate concentration of 0.9 mM, the assays were usually run in 2 mM phenylalanine for insurance. This level of phenylalanine yields the same activity as does the initial plateau value of the substrate. p-Chlorophenylalanine at a molar ratio of 16:1 with respect to phenylalanine inhibits the enzyme by 75%. Furthermore, this analog is not converted to tyrosine. Additional investigations into the nature of this inhibition were not carried out.

Phenylalanine hydroxylase activity as a function of $DMPH_4$ concentration A $DMPH_4$ dependency curve is shown in Figure 8. It will be noted that this curve bears a strong resemblance to that for the phenylalanine curve. The concentration of $DMPH_4$ employed in assays was 0.32 mM.

Figure 7. Phenylalanine hydroxylase activity as a function of phenylalanine concentration. Varying concentrations of phenylalanine were added to the usual assay system (see Materials and Methods, section 4). Tyrosine was assayed by the paper chromatography technique (see Materials and Methods, section 4B). The values derived when a heat inactivated enzyme preparation was used in place of the active material (crude extract in this experiment) were subtracted to yield quantities of enzymatically synthesized tyrosine.

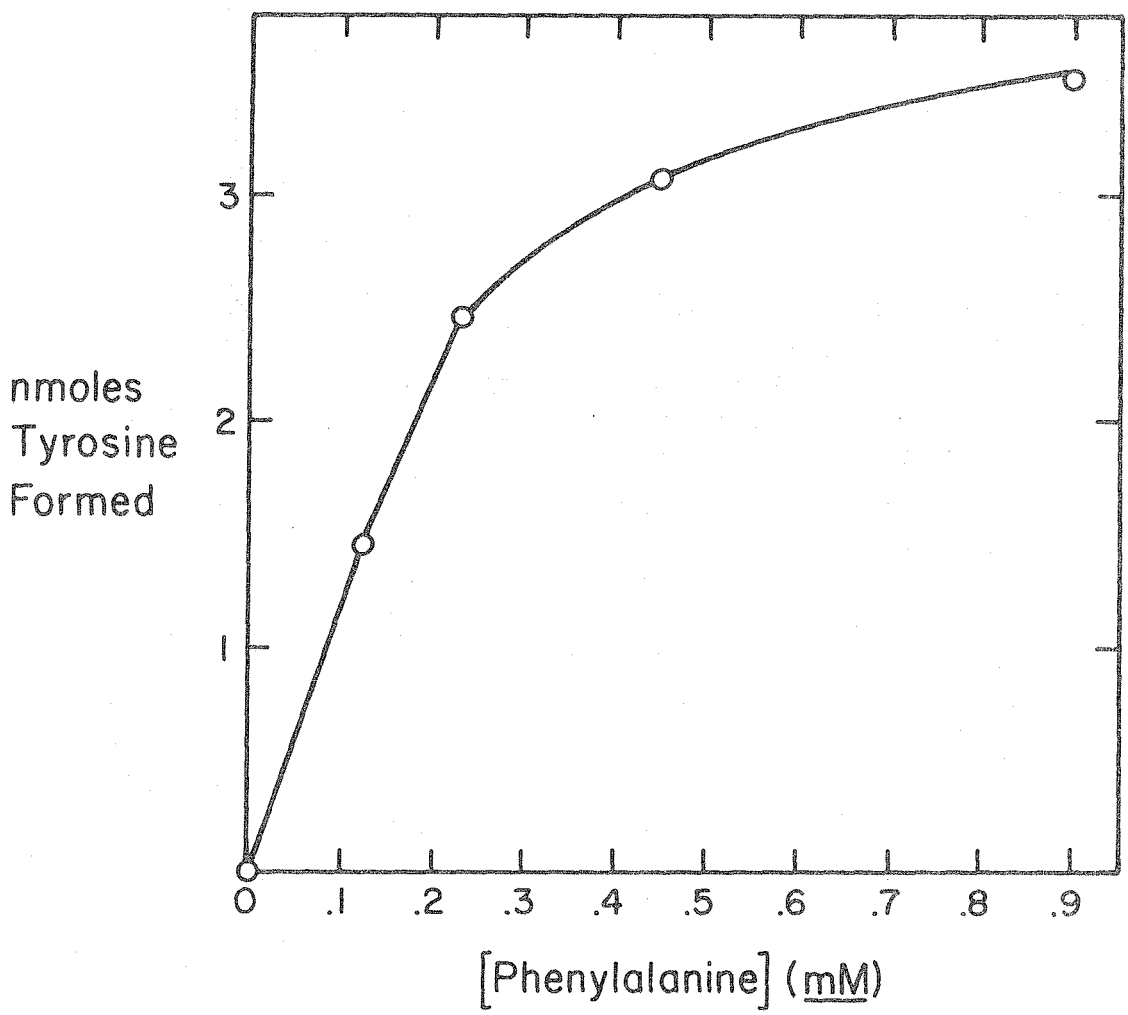
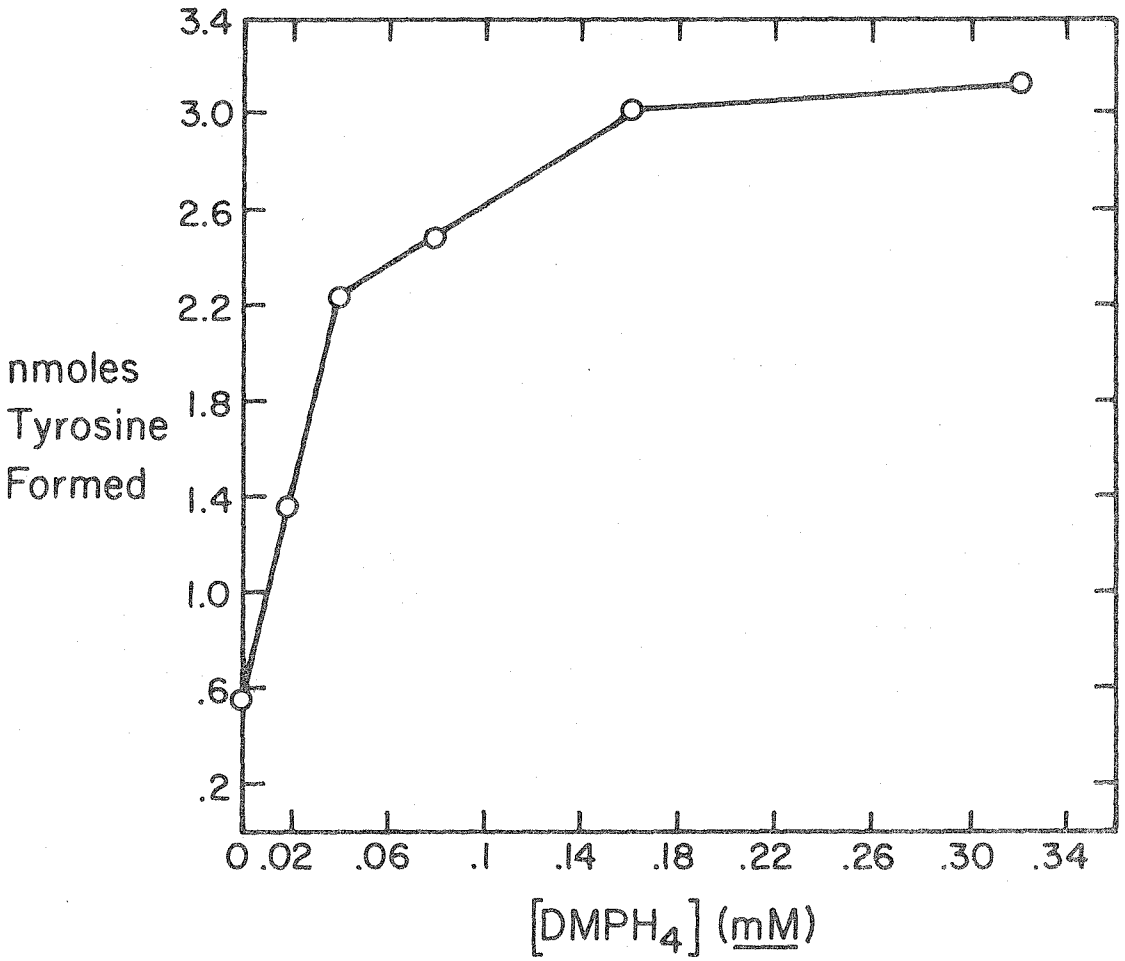

nmoles
Tyrosine
Formed
3
2
1
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
[Phenylalanine] (mM)

Figure 8. Phenylalanine hydroxylase as a function of pteridine concentration. Essentially the same conditions were used as described in Figure 7, however, here the pteridine concentration was varied. Tyrosine was assayed by the fluorescence technique (see Materials and Methods, section 4A). Substrate independent tyrosine was subtracted from those values derived with phenylalanine to yield quantities of tyrosine derived from hydroxylase activity.


3.4
3.0
2.6
2.2
1.8
1.4
1.0
.6
.2
nmoles
Tyrosine
Formed
0
.02
.06
.1
.14
.18
.22
.26
.30
.34
[DMPH4] (mM)

Phenylalanine hydroxylase activity as a function of pH Figure 9 depicts the activity of the hydroxylase at a series of varying hydrogen ion concentrations. There is a fairly broad peak of activity in the neutrality range. Although not shown, the level of substrate independent tyrosine accumulation increases at higher pH values. To counteract this effect as much as possible, the pH of most assays was maintained around 7.

Phenylalanine hydroxylase activity as a function of the age of the animal Phenylalanine hydroxylase activity was assayed *in vitro* at a number of different stages of development. The results from such an experiment are summarized in Figure 10. The activity builds up during early larval life, the first detectable activity at 48 hours past egg collection, up to a peak of activity at the time of puparium formation. Once the animal has formed the tan pupal case, the level of activity falls to less than 1 nanomole of tyrosine/mg protein, which is the limit of sensitivity of the assay. This level is maintained throughout pupal life. There may be a very small amount of enzyme at the time of emergence.

It is not known if the troughs in activity observed at 68, 105 and 120 hours are significant. More samples must be

Figure 9. Phenylalanine hydroxylase activity as a function of pH. 100 µl of enzyme (40-60% fraction - see Materials and Methods, section 7) was diluted in 0.8 ml of appropriate buffer and used in the standard assay system (see Materials and Methods, section 4). To maintain pH values in the range of 5.5-6.5, the enzyme was diluted in 0.1 M MES; for pH values of 7.0 and 7.5, the dilution was made with 0.1 M MOPS; and, for pH values of 8.0 and 8.5, 0.1 M bicine. A spot check of the diluted materials revealed adequate buffering capacities. Tyrosine was assayed by the fluorescence technique (see Materials and Methods, section 4A). Values for hydroxylase activity were derived by subtracting phenylalanine independent tyrosine formation from those derived from the complete system.

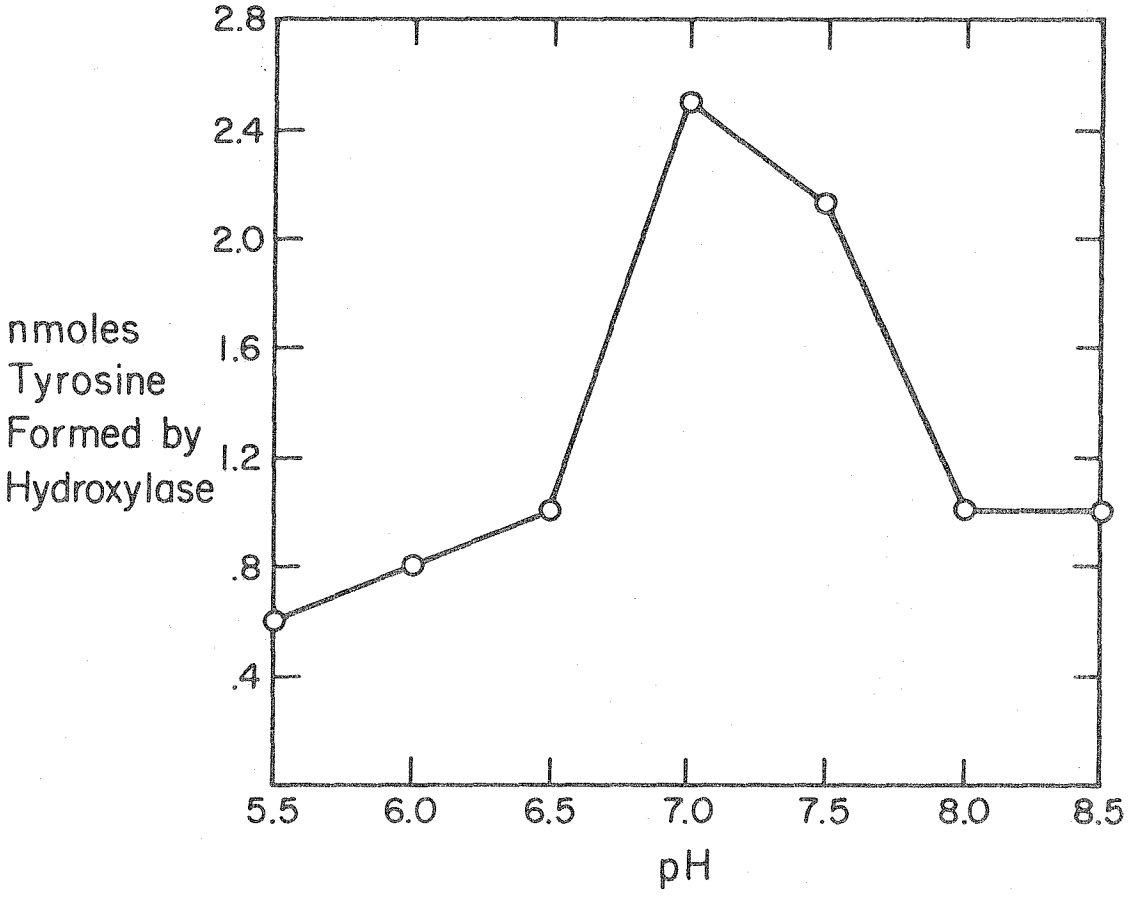
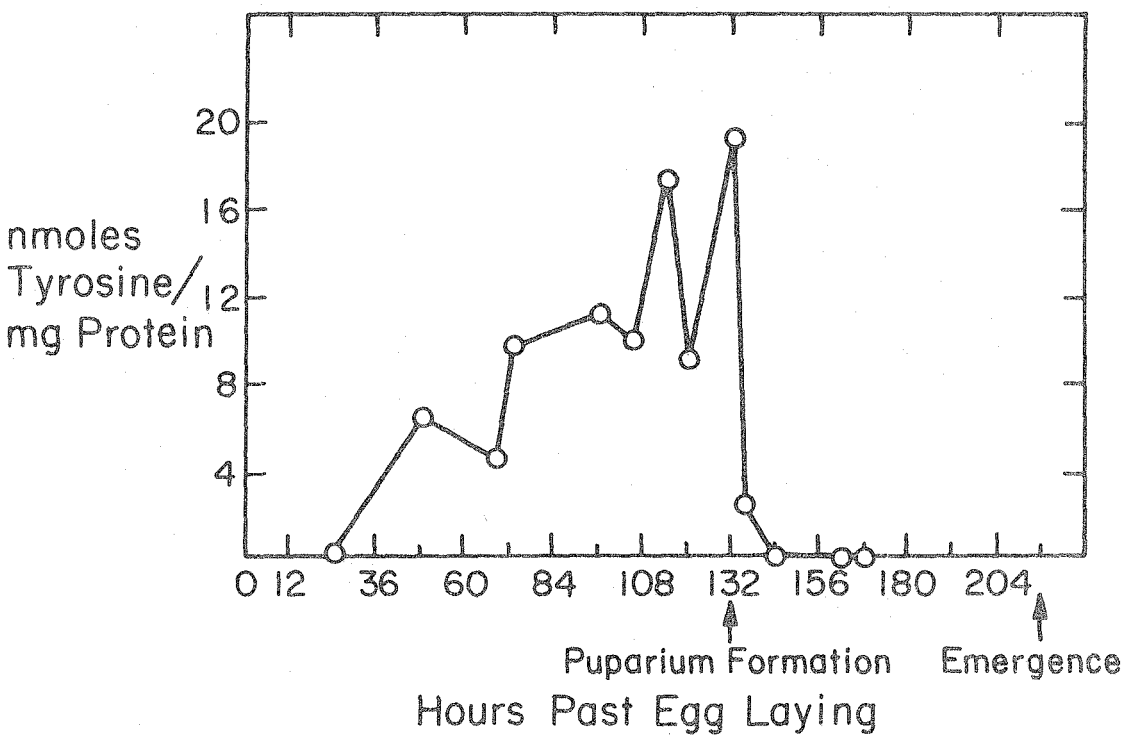

nmoles
Tyrosine
Formed by
Hydroxylase
2.8
2.4
2.0
1.6
1.2
.8
.4
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH

Figure 10. Phenylalanine hydroxylase as a function of the age of the animal. Different aged animals were collected throughout the Drosophila life cycle and extracts made of each as described in Materials and Methods, section 3. The amount of tyrosine synthesized through the action of the hydroxylase was determined by the fluorescent technique (see Materials and Methods, section 4A) and the protein content of the extracts was determined by the Lowry procedure (104).


nmoles
Tyrosine/
mg Protein
20
16
12
8
4
0 12 36 60 84 108 132 156 180 204
Puparium Formation
Emergence
Hours Past Egg Laying

taken during these time periods to establish firmly the validity of these numbers.

In order to determine if the absence of activity noted in pupal stages is due to an inhibitor, not to a lack of enzyme, an aliquot derived from these inactive preparations was included in an assay system containing active enzyme. No inhibition of hydroxylase activity occurred.

Purification of phenylalanine hydroxylase

Is the enzyme soluble or particle bound? Drosophila phenylalanine hydroxylase is a completely soluble enzyme. If larvae are extracted in 0.1M phosphate at neutral pH's, essentially all of the hydroxylase activity remains in the supernatant after a 10,000 r.p.m. centrifugation for 15 minutes. The pellet contains no activity, at least not in a form that can be detected by the usual assay methods. Similarly, most of the recoverable activity is found in the supernatant after a 40,000 r.p.m. centrifugation for 1 hour, with a very small percentage in the pellet. Appreciable levels of activity can be pelleted as a result of a 60,000 r.p.m. centrifugation for 3 hours, however, many proteins behave similarly under these conditions.

Various extraction procedures It was shown above that the amount of hydroxylase activity detected in extracts

is approximately one-tenth of that seen *in vivo*. Hence, different extraction procedures were employed in an attempt to increase the yield of the available activity.

In general, the techniques used were chosen as potential stabilizers of the hydroxylase system. As will be demonstrated shortly, this molecule is extremely unstable. Extraction in the presence of either phenylalanine (0.1M) or $DMPH_4$ (0.64 mM) was without effect on the recovery. Extraction in 40% ammonium sulfate (followed by adjustment to 60% saturation for isolation of the enzyme-see below) was without substantial effect. Since all of the above preparations were treated with Sephadex G-25 prior to assay, the possibility of loss at this step was considered. It has been found in situations such as this that a high concentration of protein in the elution buffer could obviate such losses. When bovine serum albumin at a concentration of 2 mg/ml was included in the buffer, no significant increase in hydroxylase activity occurred.

Since tetrahydropteridines are extremely light sensitive, it was thought that the level of hydroxylase activity could be elevated by protecting the assay tubes from the light. When such exigencies were met, no increase in activity was noted.

Stability of the hydroxylase in solution If a crude

enzyme preparation was allowed to incubate at 4° for three hours, a severe loss in activity was incurred. Of course, this condition was not conducive for prolonged purification procedures. A search was made for conditions which could circumvent this problem. A summary of this investigation is presented in Table 3.

When no additions were made to the preparation, approximately one half of the original activity disappeared within the three hours. Inclusion of 2.67 mM phenylalanine was without effect on this loss; however, $DMPH_4$ at a final level of 0.43 mM essentially left the activity intact during this interval. $DMPH_4$ had another effect upon this system: it inhibited the substrate independent accumulation of tyrosine. If this tyrosine is arising as a result of proteolytic activity, it is not clear how the pteridine functions to inhibit this process.

In another similar experiment, it was found that pH values above 7.0 were optimal for stability, however, substantial losses were incurred at all pH values.

It would appear, therefore, that it would be beneficial to include $DMPH_4$ in the purification procedures. There are a number of drawbacks to this scheme. The major one is that the pteridine, itself, is an extremely unstable compound, being readily auto-oxidizable and subject to photo-degradation. The incubation experiments were carried out under

Table 3

Stability of Phenylalanine Hydroxylase in Solution

conditions of incubation	% of original hydroxylase activity left after 3 hours*
in 2.67 mM phenylalanine	50
in 0.43 mM $DMPH_4$	90
no additions	50

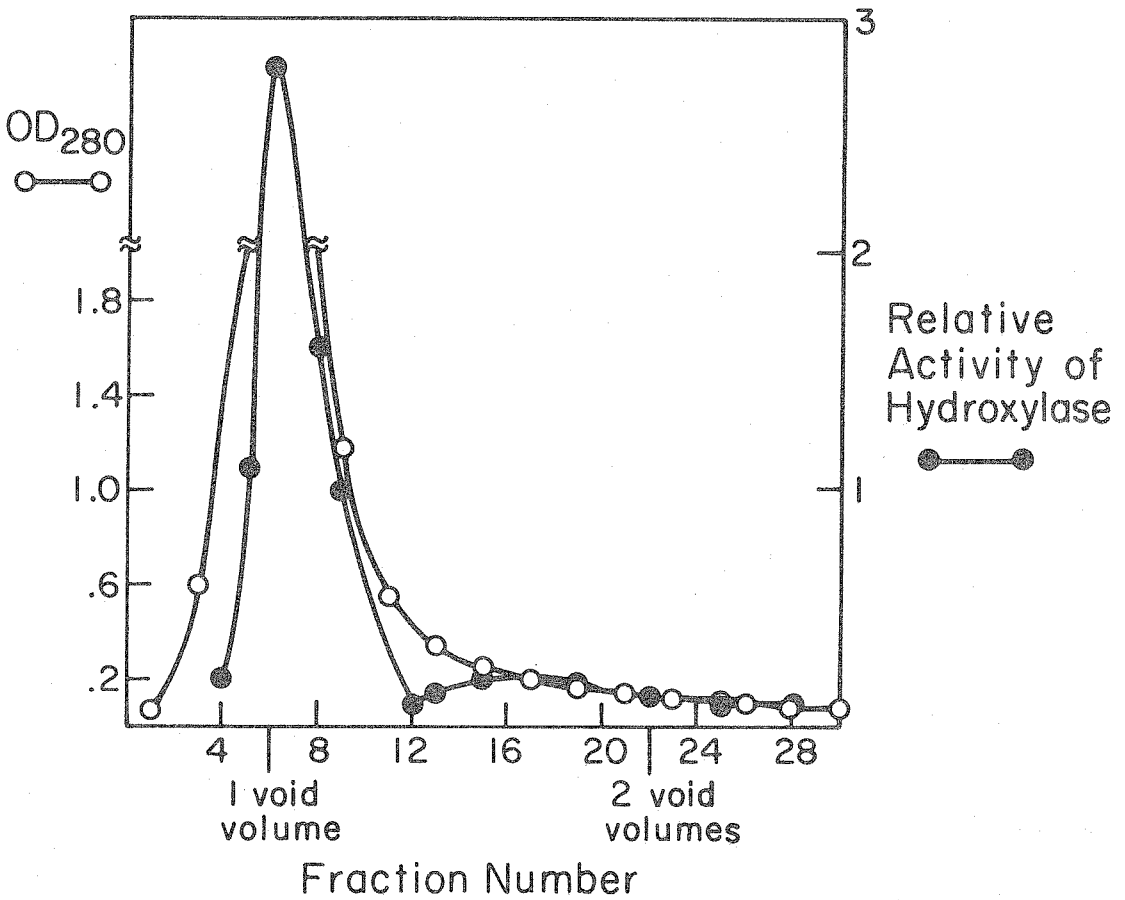
* 0.6 ml of enzyme (40-60% ammonium sulfate fraction - see Materials and Methods, section 7) was added to either phenylalanine, pteridine or buffer and incubated at 4°C for 3 hours. The tube containing pteridine was wrapped in aluminum foil to protect it from the light. To perform the assays, phenylalanine or pteridine was added to the appropriate tubes to initiate the reaction; the tube with no additions was charged with both compounds. Tyrosine was assayed by the fluorescence technique (Materials and Methods, section 4A). Values for hydroxylase derived tyrosine were arrived at by subtracting the reaction sans either phenylalanine or pteridine from the value obtained from the complete reaction.

optimal conditions, protection from air and light being afforded. It would be cumbersome to take these precautionary measures on a large scale, as would be encountered in most fractionation procedures.

Ammonium sulfate fractionation The bulk of the available phenylalanine hydroxylase activity resides in the 40-60% saturation fraction. Some activity was found in the 0-40% fraction, however, it was present in much lower quantities. This step achieves a 2-3 fold purification and also serves to concentrate the enzyme for use in subsequent procedures.

Gel filtration Phenylalanine hydroxylase activity is excluded on Bio-Gel A -0.5 and Bio-Gel P-150. A chromatogram of the latter is depicted in Figure 11. It is obvious that there is no fractionation of the hydroxylase away from contaminating proteins as a result of this step. There is a suggestion that a small fraction of the hydroxylase activity is included, but the level of activity seen is very close to the sensitivity limit of the assay. It is possible that the hydroxylase exists as an aggregate under these conditions, therefore, behaving as a large molecule. Conditions favorable for de-aggregation, such as high salt and detergents are detrimental to the activity of the enzyme and were not utilized. Phenylalanine hydroxylase is included on Bio-Gel

Figure 11. P-150 chromatography of phenylalanine hydroxylase. 1.2 ml of 40-60% fraction (see Materials and Methods, section 7) were layered onto a P-150 column (3x20 centimeters) which was equilibrated in 0.1 M phosphate, 1 mM DTT at pH 7.1. 1.5 ml fractions were collected and running time was 2 hours. Protein concentration was assayed by absorbance at 280 mμ and hydroxylase by the fluorescence method (see Materials and Methods, section 4A). The yield of hydroxylase was 32%.


OD280
3
2
1
1.8
1.4
1.0
.6
.2
Relative Activity of Hydroxylase
4
8
12
16
20
24
28
1 void volume
2 void volumes
Fraction Number

A-15 (exclusion limit of 15,000,000 daltons) as depicted in Figure 12. A 2-3 fold purification of the enzyme with a yield of approximately 40% results from this procedure. The moiety(s) responsible for substrate independent accumulation of tyrosine migrated a bit more slowly than did the hydroxylase in this particular experiment, thereby rendering a partial separation of the latter from a meddlesome contaminant.

Similar results with Bio-Gel A-1.5 (exclusion limit of 1,500,000 daltons) were obtained. The hydroxylase activity chromatographs as a rather broad band of activity at the second void volume, as can be seen in Figure 13. The degree of purification achieved is in the same range as that for the A-15 column.

Adsorption on hydroxylapetite and brushite Purification of phenylalanine hydroxylase on two types of calcium phosphate gels, hydroxylapetite and brushite, was attempted. Many different regimens of starting and elution conditions were tried, but the results were inconclusive. If activity was ever recovered, both the degree of purification and the yield were prohibitively low. Moreover, the results were inconsistent, so that this technique was without value.

Figure 12. A-15 chromatography of phenylalanine hydroxylase. 1.5 ml of 40-60% (see Materials and Methods, section 7) were layered onto a A-15 column (3x20 centimeters) equilibrated in 0.1 M Tris, 1 mM DTT at pH 7.6. 1.9 ml fractions were taken and running time was 2 hours. Tyrosine was assayed by the fluorescence technique (see Materials and Methods, section 4A) and protein by absorbance at 280 mμ. Included is the chromatography of the material responsible for substrate independent accrual of tyrosine. These values were subtracted from the values derived from the complete system to yield quantities of tyrosine derived by hydroxylase activity.

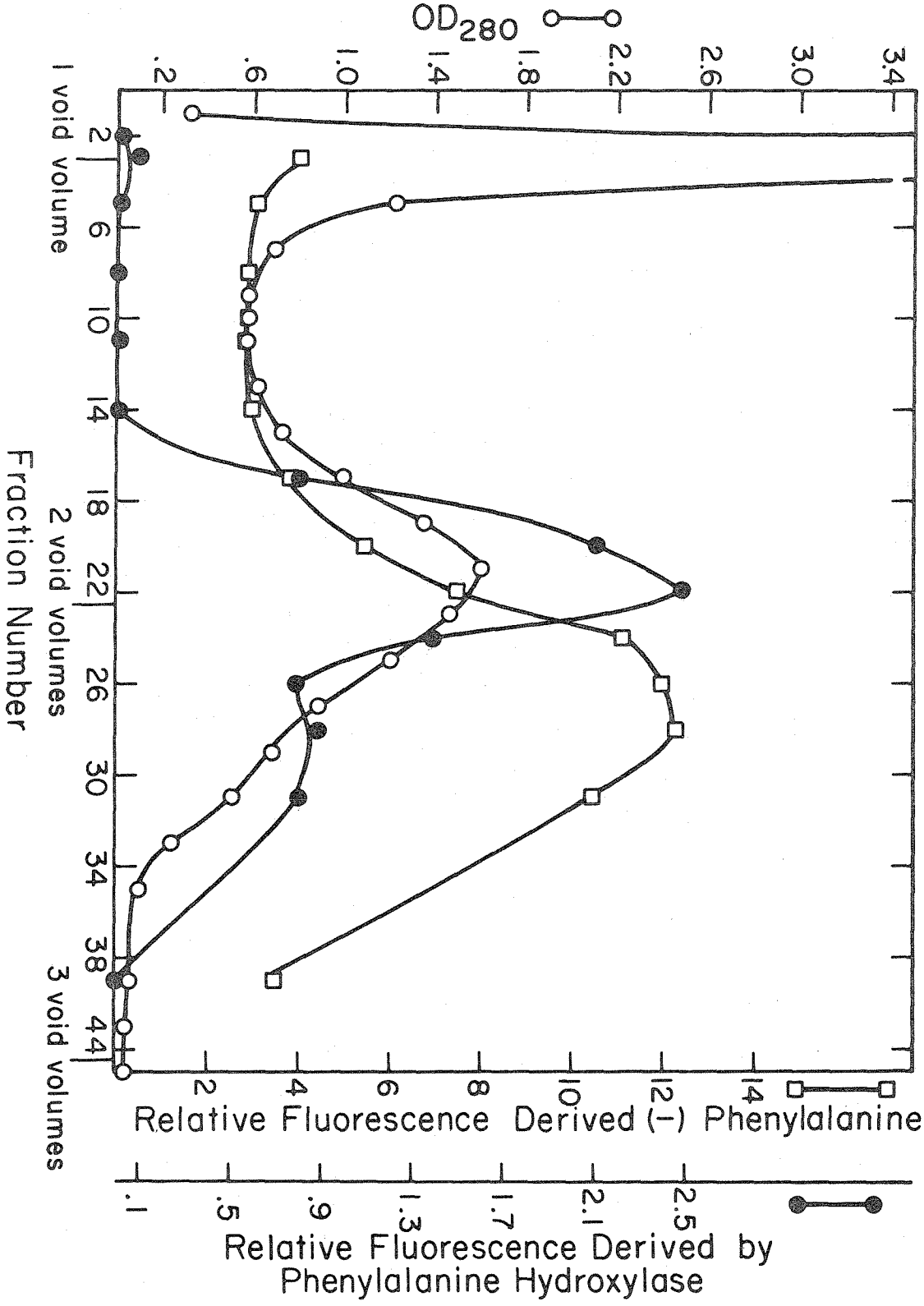
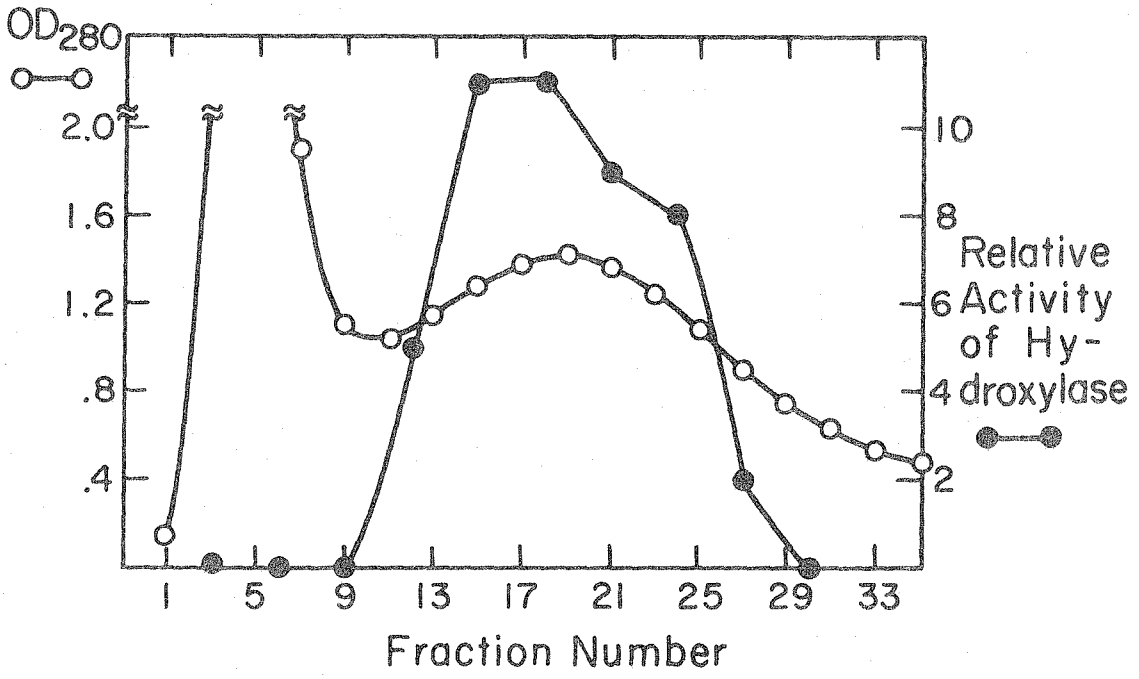

OD280
.2
.6
1.0
1.4
1.8
2.2
2.6
3.0
3.4
1 void volume
2 void volumes
3 void volumes
Fraction Number
2
6
10
14
18
22
26
30
34
38
44
2
4
6
8
10
12
14
Relative Fluorescence Derived (-) Phenylalanine
.1
.5
.9
1.3
1.7
2.1
2.5
Relative Fluorescence Derived by
Phenylalanine Hydroxylase

Figure 13. A-1.5 chromatography of phenylalanine hydroxylase. 3 ml of 40-60% fraction (see Materials and Methods, section 7) were passed over a A-1.5 column (3x40 centimeters) equilibrated in 0.1 M Tris, 1 mM DTT at pH 7.5. 2 ml fractions were collected and total running time was 2.5 hours. Protein was assayed by absorbance at 280 mμ and tyrosine by the fluorescence technique (see Materials and Methods, section 4A).


OD280
2.0
1.6
1.2
.8
.4
10
8
6
4
2
Relative Activity of Hydroxylase
1
5
9
13
17
21
25
29
33
Fraction Number

DEAE-Sephadex chromatography When preparations of hydroxylase (usually the 40-60% ammonium sulfate fraction) were treated with DEAE-Sephadex under a wide variety of starting and elution conditions, no activity was recovered.

Affinity chromatography It was thought that affinity chromatography would lend itself to purification of the hydroxylase. Preparations were treated with an agarose-phenylalanine derivative (14.5μmoles phenylalanine/ml bed-Miles Laboratories). A vast array of starting and elution schemes were tried, however, hydroxylase activity could not be recovered reproducibly under any of the conditions.

A clue to the cause of this problem is that the agarose intermediate (the activated agarose to which the phenylalanine is ultimately covalently bonded to) is charged, so that, if the reaction between these intermediates is incomplete, the resin is left with charged groups. As noted in the previous section, interactions between the enzyme and charged groups seem to result in the loss of activity.

Enzyme preparations were treated with two such intermediates, Affinose 101 (aminoethyl agarose - an anion exchanger) and Affinose 202 (a cation exchanger). The results were as expected: under a multitude of conditions, no substantial amount of activity could be recovered. It is not

reasonable to assume that when these derivatives react with the ligand to yield the final product, all of the charged groups are quantitatively converted to the agarose-phenylalanine derivative.

2. Conversion of Tyrosine to Dopa

As was discussed in the Introduction, the dopa that is destined for use in the sclerotization process can conceivably be derived via catalysis by two distinct enzymatic systems: tyrosinase or tyrosine hydroxylase. It is the purpose of this section to relate information concerning the identity of this enzyme system and the attendant means by which the hydroxylation occurs in Drosophila melanogaster.

All insect species, Drosophila among them, contain very active tyrosinase systems. In order to define the different activities which are responsible for the hydroxylation of tyrosine to dopa, the contribution of tyrosinase activity to the level of dopa observed had to be taken into account. This was often achieved by comparing the extent of the hydroxylation reaction to the dopa oxidase one.

Another line of attack in defining the activities responsible for the catalysis of this hydroxylation is fractionation of crude extracts followed by appropriate assays. These experiments also yield pertinent information concerning the feasibility of the involvement of tyrosinase in providing dopa for the sclerotization pathway.

Assay for dopa

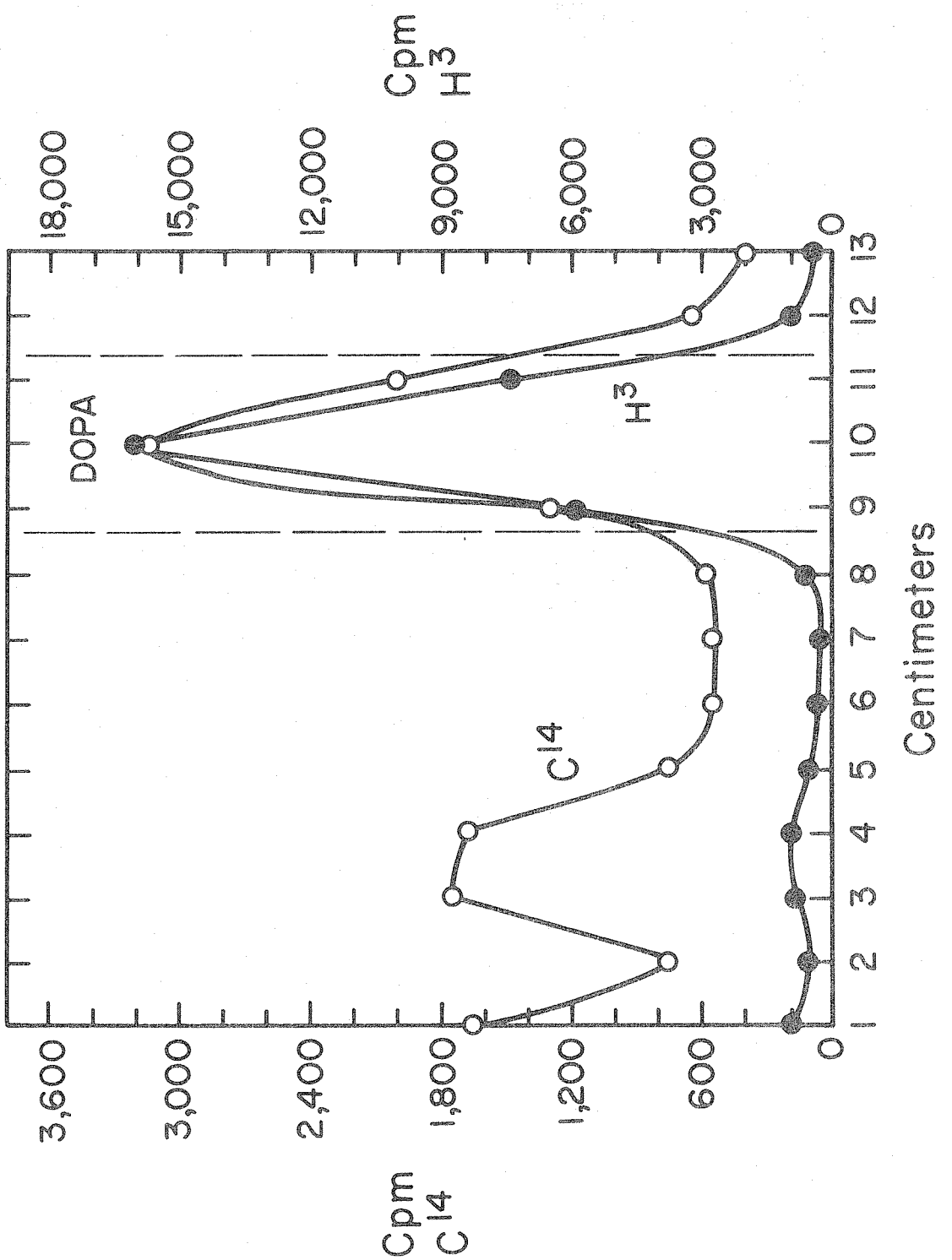
The amount of dopa generated upon incubation with C^{14}-

tyrosine with crude extracts was assayed solely by the paper chromatography technique (see Materials and Methods, section 5). Figure 14 depicts a typical chromatogram derived by this procedure. Tyrosine migrates a few centimeters to the right of dopa and is not shown in this figure. In this particular instance, tritiated dopa was run along as an internal standard. It can be seen that the C^{14} peak in the 9-12 centimeter region is coincidental with the tritium peak, thusly identifying the peak as containing authentic dopa.

Ascending chromatography of an aliquot from a reaction mixture was carried out in a chromatography system composed of n-butanol/acetic acid/water (12:3:5). The developed chromatogram displayed activity identical to that derived in the usual system - descending chromatography in n-butanol/acetic acid/water (4:1:5).

In all experiments, C^{12} dopa was run along as a standard and its position on the chromatogram assigned by its reaction with ninhydrin. Note should also be made in Figure 14 of the counts deposited at the origin of the chromatogram and the peak of activity in the 2-5 centimeter region, which has been termed peak I and will be dealt with shortly. Subsequent experiments demonstrated that both of these activities are derivable from dopa. The material at the origin represents melanin or a mixture of the precursors to the final melanin product. Observationally, there is a direct correlation

Figure 14. A chromatogram derived for a standard tyrosine hydroxylation assay. A tyrosine hydroxylation assay was carried out accoriding to Materials and Methods, section 5. After the reaction was terminated, 1.1×10^7 dpm of tritiated L-dopa (Schwarz-Mann, specific activity = 14 c/mmole) was added and a 25 µl aliquot was subjected to descending paper chromatography.


Cpm H3
18,000
15,000
12,000
9,000
6,000
3,000
0
DOPA
H3
C14
Centimeters
0 1 2 3 4 5 6 7 8 9 10 11 12 13
3,600
3,000
2,400
1,800
1,200
600
0
Cpm C14

between the number of counts derived in this region and the darkness of the area, which is a measure of melanin formation (or its precursors). No other region of the developed chromatogram displays this coloration.

Since both of these activities are derived from dopa, they must be taken into account when total hydroxylation is being measured.

Use of either a heat inactivated enzyme preparation or phosphate buffer in lieu of normally treated preparations served as controls in all enzyme assays. Appropriate subtractions were made to yield the amount of product derived by enzymatic means.

Nomenclature

A number of terms and abbreviations are used frequently in the ensuing sections and require explanations.

The term"tyrosine hydroxylase" (when set in quotation marks) refers to any system which catalyzes the conversion of tyrosine to dopa, whether it be the classical tyrosinase or a distinct tyrosine hydroxylase molecule that is not associated with the tyrosinase complex.

Tyrosinase and phenol oxidase are synonymous terms.

Dopa oxidase refers to the reaction catalyzed by phenol oxidase whereby dopa is oxidized to dopaquinone and ultimately yielding melanin.

The term origin material is a description of that material which remains at the origin of the developed chromatogram and corresponds to the product of the dopa oxidase reaction (melanin or its precursors). In many figures and tables, the quantity of origin material is expressed in nanomoles. Since the molecular weight of this material is undefined, the quantity actually refers to the nanomoles of dopa consumed to yield the product.

The following abbreviations are used extensively throughout these sections: DTT (dithiothreitol or Cleland's reagent), $DMPH_4$ (2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine), dopa (dihydroxyphenylalanine), dpm (disintegrations per minute) and cpm (counts per minute).

Properties of the Hydroxylation Reaction in Crude Extracts and Response of the Crude System to Various Chemical Perturbations

$DMPH_4$ stimulation of the reaction The major distinguishing feature associated with tyrosine hydroxylase activity is its absolute dependency upon $DMPH_4$ (89, 90). Therefore, reactions employing crude extracts derived from five day old larvae as a source of enzyme were carried out in various concentrations of $DMPH_4$. The results from such an experiment are depicted in Table 4.

Many striking features arise from the data in this Table.

Table 4

The Effect of $DMPH_4$ on the Tyrosine Hydroxylation Reaction Catalyzed by a Crude Enzyme Preparation

	nmoles product*	final ($DMPH_4$) 8 mM	0.8 mM	0.4 mM	0
	origin material	0.03	0.14	0.28	0.04
+enzyme	peak I material	0	0.26	0.33	0
	dopa	1.78	1.0	0.79	0
	origin material	0.08	0.17	0.13	0.11
-enzyme	peak I material	0.15	0.34	0.24	0.17
	dopa	0.76	0.88	0.57	0.32

* Varying concentrations of pteridine were added to the standard tyrosine hydroxylation assay (see Materials and Methods, section 5) where tyrosine was present at a concentration of 0.1 mM. The values displayed for the enzymatic reaction were derived by subtracting the respective non-enzymatic values from the overall values.

The highest concentration of $DMPH_4$ severely inhibits the synthesis of origin material, whereas the accumulation of dopa is tremendously enhanced in comparison with the reaction where the pteridine is absent. The two lower concentrations of $DMPH_4$ also enhance the accrual of dopa over the no-addition case, however, in both cases, synthesis of origin material is also stimulated, the lowest level of $DMPH_4$ being the most efficient in this latter consideration.

Furthermore, a high concentration of pteridine completely inhibits the accrual of peak I material, whereas the lower levels offer enhancement. It should be noted that in experiments similar to the one depicted in Table 4, that in the absence of $DMPH_4$, peak I is seen to accumulate occasionally, however, never to the same degree as when a small amount of pteridine is present.

This set of data also reveals that there is a very active nonenzymatic hydroxylation of tyrosine which is $DMPH_4$ dependent. In this particular experiment, the appearance of peak I is also dependent upon the presence of pteridine , however, this phenomenon was not consistently observed.

The differences seen in the synthesis of origin material which is derived non-enzymatically are slight and it is not clear that any significance can be attached to them.

Obviously the inclusion of a sufficiently high concentration of $DMPH_4$ serves two functions: blockage of dopa oxidase

activity, as reflected by the dimunition in activity observed at the origin, and, secondly, enhancement of dopa accumulation.

It does not follow that merely because the hydroxylation reaction is stimulated by the presence of $DMPH_4$ that the synthesis of dopa is due solely to the activity of a tyrosine hydroxylase with the pteridine functioning as a classical cofactor. Many of the experiments that will be presented subsequently deal with evaluating the notion that this dopa is derived from such a source. The reaction of the system to $DMPH_4$ under various conditions will be used as a measure of this enzyme's potential contribution to dopa synthesis.

Additionally, the manner in which pteridine inhibits dopa oxidase activity will be taken up in a later section.

The nature of peak I As noted in the previous two sections, when a crude enzyme preparation is incubated with C^{14}-tyrosine, a peak of radioactivity appears in the chromatogram a few centimeters away from the origin. Peak I can be derived from either tyrosine or the product of its hydroxylation, dopa.

C^{14}-dopa was incubated with the extract under identical conditions as when tyrosine is used and it was found that the material in peak I did arise from dopa and its level of accumulation was stimulated by the addition of the pteridine.

It should be pointed out that under the standard incubation conditions (see Materials and Methods, section 5), there are no additional synthetic abilities (utilizing either tyrosine or dopa as substrate) as reflected by the region of the chromatograms to the right of dopa and tyrosine.

This same result was achieved qualitatively in a number of experiments, however, the degree of stimulation by $DMPH_4$ was highly variable.

The identity of the material responsible for peak I is unknown. The possibility that the material represents peptides, which chromatograph in this region, was investigated by carrying out a reaction in the presence of 0.3 mg/ml cycloheximide. This inhibitor of ribosome-mediated peptide synthesis had no effect on the level of peak I.

Peak I was not consistently observed in the chromatograms. Initially, both the appearance and amount of activity derived were reasonably reproducible. However, for no apparent reason, the activity was lost, and, although the original extraction and assay conditions were rigorously adhered to, the presence of peak I remained irreproducible. Consequently, no further direct experiments were performed on the elucidation of the nature of the material, however, some information was accrued indirectly in a number of experiments, and the pertinent data are presented when applicable.

When it was present, it had to be taken into account when total hydroxylation of tyrosine was being measured because it is derived from the product of the reaction.

The effect of a decarboxylase and monoamine oxidase inhibitor on the hydroxylation reaction NSD-1015 (3-hydroxybenzylhydrazine hydrochloride), a potent decarboxylase and monoamine oxidase inhibitor, was included in the standard assay system at a final concentration of 1.78mM. The presence of this reagent had no effect upon the level of dopa and origin material accrued. It should be added that these reactions were carried out in the presence of 0.89mM DTT, a condition which could possibly mask any effect which this compound might produce.

If it is assumed that DTT has no effect upon the expression of NSD-1015, it is apparent that the crude extracts do not possess decarboxylase or monoamine oxidase activities in sufficiently high concentration to metabolize to any noticeable extent either the substrate tyrosine or the product dopa.

The effect of DTT on the hydroxylation of tyrosine catalyzed by crude enzyme preparations The original experiments on this system were carried out in the presence of 1-2mM DTT. The purpose of this inclusion was to prevent darkening of the extracts due to the activity of phenol

oxidase. DTT used at these concentrations was very effective in this respect. Since DTT has such a marked inhibitory effect upon dopa oxidase, its effect upon the "tyrosine hydroxylase" activity of the crude enzyme preparations was measured and the results are displayed in Table 5.

In the situation where $DMPH_4$ at a final concentration of 0.4mM was utilized, DTT inhibits the synthesis of dopa by 43%, whereas the accumulation of origin material is inhibited by 60%. In a separate experiment where dopa was used as substrate, this latter inhibition approached 80%. DTT also inhibits the initial rates of the two reactions in the same fashion.

The probable reason for this differential inhibition of the two activities is that the reduction potential of DTT is -0.332 (99), a property which allows for the reduction of dopaquinone back to dopa. Even though dopa oxidase may still be active in dehydrogenating dopa, the level of activity observed will be too low since the accumulation of origin material is dependent upon the presence of dopaquinone.

Although DTT serves a useful function in arresting the accrual of dopaquinone, it does produce deleterious effects upon the "tyrosine hydroxylase" activity.

In this particular experiment, DTT also completely inhibited the synthesis of the material in peak I. In the

Table 5

The Effect of DTT on the Tyrosine Hydroxylation Reaction Catalyzed by a Crude Enzyme Preparation

product measured* (in nmoles)	-DTT $+DMPH_4$	-DTT $-DMPH_4$	+DTT $+DMPH_4$	+DTT $-DMPH_4$
origin material	0.10	0.026	0.04	0.019
Peak I material	0.19	0	0	0
Dopa	1.2	0	0.68	0

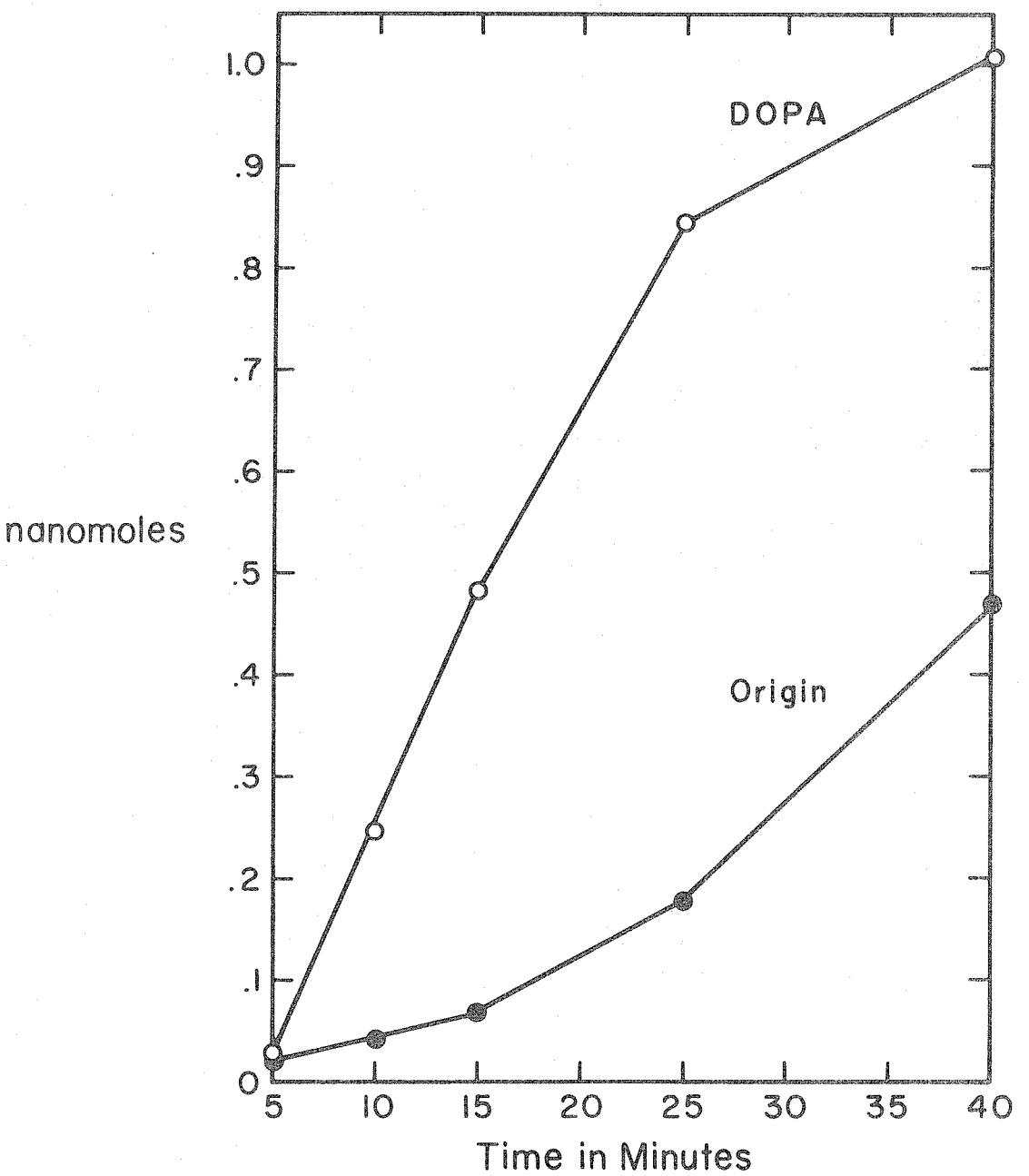
* The standard hydroxylation assay, as delineated in Materials and Methods section 5, was performed in the presence of 0.5 mM DTT and in its absence. Tyrosine was present at a concentration of 0.1 mM and pteridine, when present, at 0.4 mM. The appropriate non-enzymatic values were subtracted to yield quantities of products derived enzymatically.

preliminary experiments, DTT was always present in the assays, and peak I was observed to be present, however, there was usually more dopa oxidase and "tyrosine hydroxylase" activity in the experiments than was present in the one described in Table 5. Hence, the ratio of DTT to enzymatic activity appears to be critical with respect to the ability of the responsible enzyme to synthesize the material.

Kinetics of the dopa oxidase and "tyrosine hydroxylase" reactions catalyzed by crude enzyme preparations The rates of synthesis of origin material and dopa were determined over a forty minute interval and the results are summarized in Figure 15.

Dopa is accrued at a rate of 0.045 nanomoles/minute during the first 15 minutes of the reaction, whereupon the rate diminishes to 0.036 nanomoles/minute during the ensuing 10 minute interval, a diminution equivalent to 0.0090 nanomoles/minute. Origin material is accumulated at a rate of 0.004 nanomoles/minute for the first 15 minutes of the reaction, whereupon the rate jumps to 0.0114 nanomoles/minute during the next 10 minutes. Hence, the rate of accrual of origin material (depletion of dopa) is larger than the diminution in rate of synthesis of dopa, suggesting that dopa arises via at least two distinct pathways.

Figure 15. Kinetics of appearance of origin material and dopa in reactions catalyzed by crude larval extracts. A reaction tube was set up that contained tyrosine and pteridine at final concentrations of 0.4 mM. 25 µl aliquots were withdrawn at designated times and heated at 70°C and subjected to paper chromatography in the usual manner (see Materials and Methods, section 5).


nanomoles
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
DOPA
Origin
5
10
15
20
25
30
35
40
Time in Minutes

In an experiment similar to this one, it was found that peak I material was accumulated in a linear fashion for 25 minutes, whereupon the rate severely tapered off. DTT reduced the initial rate of synthesis.

The relative abilities of extracts derived from different aged animals to catalyze the "tyrosine hydroxylase" and dopa oxidase reactions "Tyrosine hydroxylase" and dopa oxidase levels of activity were assayed in various aged animals as delineated in Table 6. The younger animals (72, 84 and 96 hours) demonstrated active "tyrosine hydroxylase" systems with very minimal dopa oxidase activities. The older animals (110, 130, 165 and 186 hours) displayed very active dopa oxidase systems and the ratio of the two activities approached 1. There was one potential drawback to these assays in that the amount of substrate (tyrosine) present could have been limiting, although only about 75% was converted to products in the most active reactions.

When these assays were repeated (using the same preparations used in the original ones), both enzymes displayed much more activity than previously, suggesting that the preparations "aged". In fact, the "tyrosine hydroxylase"/dopa oxidase ratio for the 96 hour preparation approached a value of 1, and the ratios for the 72 and 84 hour decreased, but were still greater than were the values for the 110-186 hour

Table 6

The Relative Abilities of Crude Extracts Derived From Various Aged Animals to Catalyze the Hydroxylation of Tyrosine and Oxidation of Dopa Reactions

hours past egg collection	"tyrosine hydroxylase" dopa oxidase*
72	61
84	43
96	16.8
110	0.83
130	0.85
186	1.3

* Different aged animals were collected and extracts made of each at a volume to weight ratio of 8:1 according to Materials and Methods, section 3. The level of dopa derived in the presence of 8 mM pteridine reflects the level of "tyrosine hydroxylase" and the counts deposited at the origin (in the absence of the pteridine) reflect the level of dopa oxidase activity.

preparations.

This "aging" phenomenon could be a reflection of the latency of tyrosinase activity in crude extracts (111). The duration of the inactive period is dependent upon the concentration of the extract. Two extracts of 90 hour larvae were derived, one at a volume to weight ratio of 9:1, the other at 5:1. Both preparations were allowed to incubate without substrate for 45 minutes at 4°C prior to the assay. The dilute extract yielded a "tyrosine hydroxylase"/ dopa oxidase value of 15, while the concentrated preparation gave a value of 4.5. Hence, the ratio of the two activities depends upon the concentration of the enzyme preparation, which is probably a reflection of the efficiency of the activation process for tyrosinase.

The distribution of dopa oxidase and "tyrosine hydroxylase" activities upon fractionation of crude extracts

This section of the Results deals with fractionation of the crude larval extracts in an attempt to define a distinct tyrosine hydroxylase activity, one which is devoid of a dopa oxidase function, if, in fact, such a molecule exists. Some of the data presented in the previous section suggest that another enzyme, in addition to tyrosinase, may be involved in the synthesis of dopa.

Ammonium sulfate fractionation of crude extracts

Crude larval extracts were subjected to ammonium sulfate fractionation to study further the nature and inter-relationships of the enzymes involved in these reactions. Three fractions were obtained: material insoluble in the 0-40% saturation range, material insoluble in the 40-60% range and a combination of the two fractions. The results from such an experiment are delineated in Table 7.

The 0-40% fraction contains enzymes necessary for the synthesis of all three metabolites, whereas the 40-60% fraction is devoid of all such activities. However, when equal volumes of the 2 fractions are mixed, all three activities are enhanced: dopa synthesis by a factor of 5, origin material by 6 and peak I material by 3 (the latter two sans $DMPH_4$). Apparently each enzyme(s) responsible for the synthesis of the three metabolites requires at least two components for full activity.

The above assays were carried out in the presence of 1mM DTT, which from the above discussion, proved to be inhibitory to both the "tyrosine hydroxylase" and dopa oxidase activities. A similar type of experiment was performed utilizing high concentrations of $DMPH_4$ to inhibit dopa oxidase activity, which was measured in these assays by merely deleting the pteridine from the reaction mixtures. Quali-

Table 7

Ammonium Sulfate Fractionation of Crude Larval Extracts

fraction* + conditions	NANOMOLES PRODUCT origin material	peak I material	dopa
(0-40%)+$DMPH_4$	0.49	0.85	1.22
(0-40%)-$DMPH_4$	0.15	1.12	0
(40-60%)+$DMPH_4$	0	0	0
(40-60%)-$DMPH_4$	0	0	0
M+$DMPH_4$	1.37	6.6	6.25
M-$DMPH_4$	0.94	3.3	0.71

* A crude larval extract derived from 4 day old animals (concentration of extract = 10:1, v/w) was subjected to ammonium sulfate fractionation in essentially the same manner as described in Materials and Methods, section 7. The 0-40% pellet was taken up in 0.1 M phophate, 2 mM DTT at a volume equal to 2% of the original volume of the extract, while the 40-60% pellet was suspended in the same buffer at a volume equivalent to 4% of othe original volume. Both fractions were subjected to Sephadex G-25 chromatography prior to assay. For the assay, both fractions were diluted 1:2 in the phosphate-DTT buffer. Equal volumes of both fractions were combined to yield the M preparation. Tyrosine was present at a final level of 0.1mM, pteridine at 0.43 mM and DTT at 1.14 mM. A heat inactivated enzyme preparation served as a control and the values derived were subtracted to yield quantities derived enzymatically.

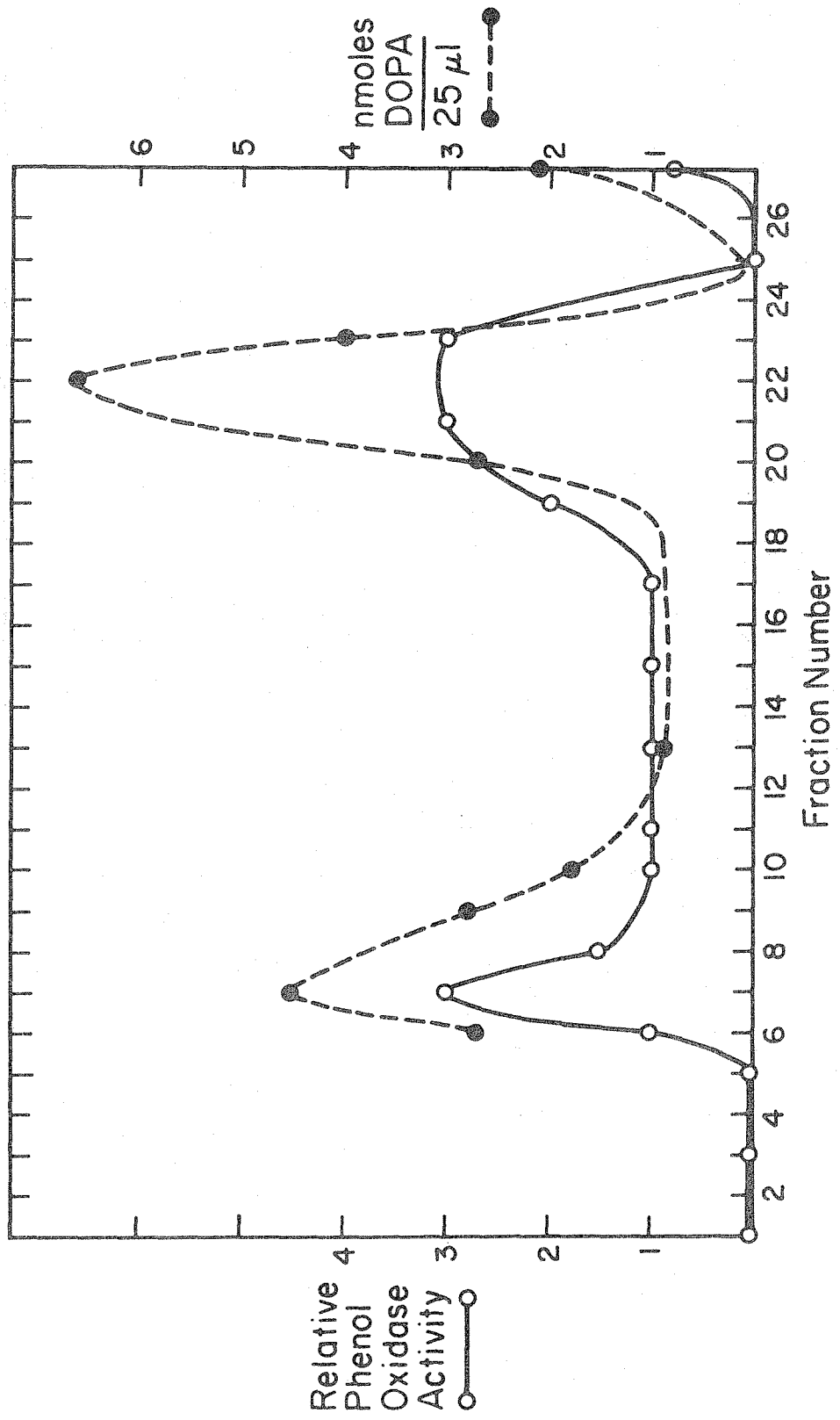
tatively, the results were similar to those expressed in Table 7, however, upon mixing equal volumes of the two fractions, the dopa oxidase function became much more active than the "tyrosine hydroxylase" activity.

Therefore, in the situation where the dopa oxidase activity of the preparation is relatively low (Table 7), the enzymes responsible for the synthesis of dopa and origin material behave similarly, that is, they are both "activated" to approximately the same degree when the two ammonium sulfate fractions are combined. However, when the preparations are rich in dopa oxidase activity, the synthesis of origin material is substantially enhanced in comparison to the "tyrosine hydroxylase" activity.

Sucrose gradient centrifugation To investigate further the relationship between phenol oxidase and the enzyme(s) responsible for the hydroxylation of tyrosine, use was made of the former's well-defined behavior in equilibrium density sucrose gradients. If activated phenol oxidase is subjected to equilibrium centrifugation in a dense sucrose medium, a number of distinct bands of activity are derived which are dependent upon genetic constitution and stage of development (87).

A sample of activated phenol oxidase derived from ebony displays two bands of activity upon centrifugation as depicted in Figure 16. This graph also demonstrates that the only

Figure 16. Sucrose gradient centrifugation of activated phenol oxidase derived from ebony. Activated phenol oxidase was subjected to equilibrium density sucrose gradient centrifugation, essentially employing the protocol of Mitchell, et al. (87). A 0-56% ammonium sulfate fraction, after passage over Sephadex G-25, was allowed to activate for 3.5 hours at 4°C prior to the centrifugation, which proceeded for 16 hours at 14,000 r.p.m. 1 ml fractions were collected and dopa oxidase activity and "tyrosine hydroxylase" activity were assayed for according to Materials and Methods, sections 6 and 5, respectiviely. In the former assay, dopa was utilized as substrate, while in the latter, tyrosine was utilized soley at a concentration of 0.115 mM. Pteridine was present also at a final concentration of 9.15 mM.


nmoles DOPA / 25 µl
Relative Phenol Oxidase Activity
Fraction Number

material that is capable of catalyzing the hydroxylation of tyrosine co-purifies with the dopa oxidase activity. Although not depicted on this graph, in similar experiments, the region between the two main peaks of dopa oxidase activity is also essentially devoid of "tyrosine hydroxylase" activity.

The synthesis of dopa catalyzed by these two bands of phenol oxidase is dependent upon the presence of $DMPH_4$. This situation is elaborated upon in the experiment described in Table 8. The most noteworthy feature of this experiment is that this reaction responds to pteridine in the same manner as does the reaction catalyzed by crude enzyme preparations. Furthermore, this material also accounts for a $DMPH_4$ dependent accumulation of peak I material.

Because of the extreme density of the sucrose gradients employed and the unique aggregation property of activated phenol oxidase, this heavy banded material, in particular, is relatively free from contaminating enzymes (107). Hence, these data suggest that pure phenol oxidase, replete with dopa oxidase activity, hydroxylates tyrosine in much the same manner as does an entire crude extract.

Some properties of the banded phenol oxidase possessing "tyrosine hydroxylase" activity

Since it appears from the foregoing discussion that

Table 8

$DMPH_4$ Stimulation of the Tyrosine Hydroxylation Reaction Catalyzed by Heavy Band Material

product measured* (in nmoles)	final ($DMPH_4$) 0.8 mM	0.2 mM	0.1 mM	0
origin material	0.7	1.8	1.68	0.76
peak I material	0.46	0.28	0.19	0.11
dopa	4.0	2.2	1.1	0

* Varying concentrations of pteridine were added to the standard assay system (see Materials and Methods, section 5) where heavy band tyrsoinase was utilized as the source of enzyme. Tyrosine was present at a final concentration of 0.21 mM. A non-enzymatic reaction was also performed in the presence of 0.8mM pteridine and the values derived subtracted from those derived from the over-all reaction to yield levels of enzymatically synthesized metabolites.

banded phenol oxidase is capable of utilizing $DMPH_4$ to stimulate the tyrosine hydroxylase reaction, a series of experiments was undertaken to explore the nature of this reaction and the relationship between the "tyrosine hydroxylase" and dopa oxidase activities in the complex. The experiments were designed to evaluate the potential involvement of, what appears to be, pure tyrosinase in providing dopa for the sclerotization process.

Relative use of tyrosine and dopa as substrates by both bands of phenol oxidase activity In assaying fractions across the sucrose gradients described above, a trend emerged whereby the heavy band material appeared, in general, to be less able to catalyze the tyrosine→origin material reaction than was the light material.

The heavy and light banded phenol oxidases were assayed for their relative abilities to utilize the two substrates, which were maintained at equal concentrations, and the results are summarized in Table 9. It should be noted that all of these assays were carried out in the absence of pteridine so that a "pure" tyrosinase level of activity is being measured.

If the first two reactions (tyrosine→dopa, dopa→ origin material) are compared, it is seen that these activities exist in the same proportion, one band in relation to the other

Table 9

Relative Use of Tyrosine and Dopa as Substrates by the Heavy and Light Banded Tyrosinases

enzyme source	reaction*	nmoles product
	tyrosine ⟶ dopa	2
heavy band	dopa ⟶ origin	19
	tyrosine ⟶ origin	2
	tyrosine ⟶ dopa	1.4
light band	dopa ⟶ origin	11.7
	tyrosine ⟶ origin	3.5

* Both heavy and light banded material were used to catalyze 2 reactions, one utilizing tyrosine as substrate, the other using dopa. When tyrosine was used, the reactions tyrosine ⟶ dopa and tyrosine ⟶ origin material were scored, whereas when dopa was used, the dopa ⟶ origin material reaction was measured. Tyrosine was present at a final concentration of 0.118 mM and dopa at 0.11 mM. The standard assay procedure was employed (see Materials and Methods, section 5) except that pteridine and DTT were absent.

(that is,

$$\left.\frac{\text{tyrosine}\rightarrow\text{dopa by H}}{\text{tyrosine}\rightarrow\text{dopa by L}} = 1.4 \cong \frac{\text{dopa}\rightarrow\text{origin by H}}{\text{dopa}\rightarrow\text{origin by L}} = 1.6\right),$$

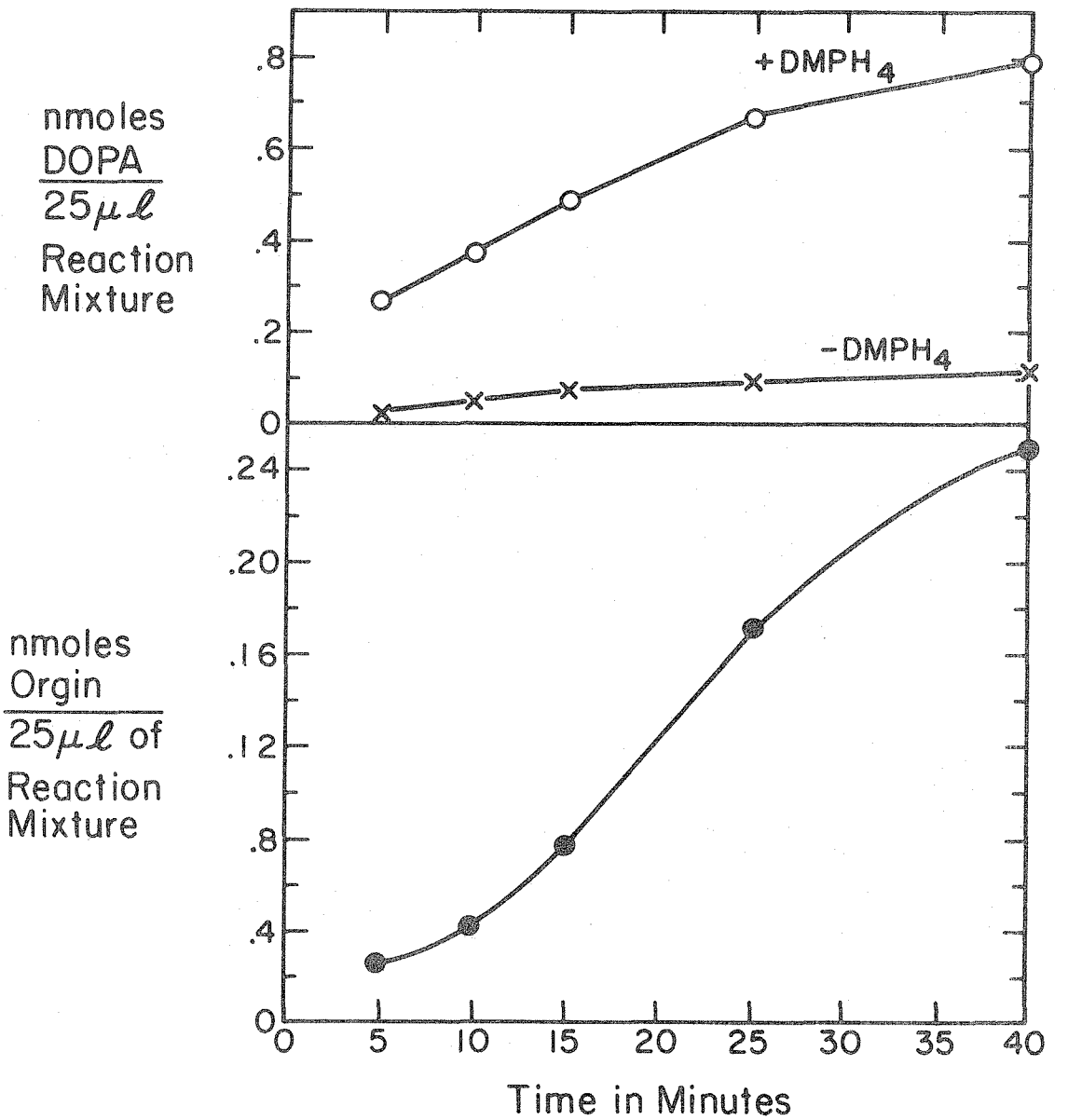
however, the reaction tyrosine→origin material is much more active when catalyzed by the light banded material.

Hence, even though the heavy band material is capable of catalyzing the dopa oxidase reaction (dopa→origin material), it is relatively deficient in its ability to catalyze the tyrosine→origin reaction. This situation could be significant in a consideration of a tyrosinase's involvement in providing free dopa for the sclerotization pathway, for this species of tyrosinase (heavy band) may be more likely to allow the newly synthesized dopa to "escape" from further oxidation processes.

Kinetics of the dopa oxidase and "tyrosine hydroxylase" reactions catalyzed by heavy band material In order to elucidate further the relationship between the dopa oxidase and "tyrosine hydroxylase" functions in banded tyrosinase, the time courses for the hydroxylation of tyrosine and synthesis of origin material were determined, as depicted in Figure 17.

In the presence of approximately 6 mM $DMPH_4$, a concentration which completely blocks the expression of dopa oxidase, dopa is accumulated at a linear rate for 25 minutes, where-

Figure 17. Kinetics of the reactions catalyzed by heavy band phenol oxidase. Two reaction tubes were set up: one containing pteridine at a final concentration of 6.4mM and the other in the absence of this factor. At appropriate time points, 25 µl aliquots were withdrawn, heated at 70°C for a few minutes and then streaked onto pre-cut strips of Whatman 3MM paper as described in Materials and Methods, section 5. The final concentration of tyrosine in both tubes was 0.08 mM. The top two curves depict the time course for dopa accrual in the presence and absence of pteridine. The bottom curve represents the kinetics of origin material synthesis in the absence of the pteridine. In the presence of 6.4 mM pteridine, very little origin material is accumulated. The ordinate describes the number of nanomoles of dopa that have accumulated at the origin in the form of melanin or its precursors.


nmoles DOPA / 25μl Reaction Mixture
+DMPH4
-DMPH4
.8
.6
.4
.2
0
nmoles Orgin / 25μl of Reaction Mixture
.24
.20
.16
.12
.8
.4
0
0
5
10
15
20
25
30
35
40
Time in Minutes

upon the rate diminishes over the ensuing 15 minute interval. This diminution may arise because of limiting amounts of substrate, although at the 40 minute point, only 40% of the available substrate has been converted to dopa. In the absence of the pteridine, dopa is accumulated very slowly over this 40 minute interval.

Graph b in this Figure demonstrates the sigmoidal nature of the kinetics of the synthesis of origin material, a situation that has been described previously (64,75).

Hence, the dopa oxidase and "tyrosine hydroxylase" activities (when assayed in the presence of $DMPH_4$) possess distinguishing kinetic features. Furthermore, the rate of the tyrosine hydroxylation reaction and the final level of dopa achieved over the 40 minute period are tremendously enhanced in the presence of the pteridine.

The effect of excess dopa upon the hydroxylation reaction catalyzed by banded phenol oxidase Standard hydroxylation assays were carried out in the presence of excess C^{12} dopa (20 fold molar excess) for two purposes: to explore possible end product inhibition of the hydroxylation reaction and, secondly, to evaluate the possibility of the existence of shared active sites for tyrosine and dopa in the tyrosinase complex. The results from this experiment are summarized in Table 10.

Table 10

The Effect of Excess Dopa on the Hydroxlation Reaction Catalyzed by Heavy Band Phenol Oxidase

	nmoles product	+dopa +$DMPH_4$	-dopa +$DMPH_4$	+dopa -$DMPH_4$	-dopa -$DMPH_4$
+enzyme	origin material	0	0	0.1	0.1
	dopa	1.06	1.95	1.0	0.16
-enzyme	origin material	0	0	0	0.017
	dopa	0.56	0.74	0.25	0.24

* Heavy band phenol oxidase was incubated in the presence of 2 mM C^{12}- D,L-dopa in the presence and absence of 8 mM pteridine and activity was assayed for in the usual manner (see Materials and Methods, section 5). Tyrosine was present at a final concentration of 0.1 mM, hence the dopa is present at a 20 fold molar excess. The values derived in the non-enzymatic reaction are also included, however, they have been subtracted appropriately to yield true enzymatically synthesized values.

A number of salient features emerge from the data in this Table. In the presence of $DMPH_4$, the excess dopa inhibits the accumulation of C^{14}- dopa by 46.5%. To verify that this effect was not the result of a chromatography artifact introduced by the high concentration of dopa, two reactions were carried out, one in the presence of 2mM dopa, the other sans dopa. After the reaction was terminated in the usual manner, dopa was added to the latter tube, buffer to the former. The subsequent work-up revealed that the excess dopa had no effect on the chromatography.

Dopa, in the absence of $DMPH_4$, stimulated the synthesis of more dopa over the situation where both compounds were absent from the reaction mixture. This reaction was enhanced by a factor of 6.4 whereas the pteridine alone stimulated the reaction by a factor of 12.2 (in comparison with the case where both are deleted).

These assays were repeated with a preparation which contained more dopa oxidase activity than what was used in the above experiment, and it was found that the excess dopa, in the absence of $DMPH_4$, inhibited the synthesis of origin material (with tyrosine as substrate) by 65%.

Both bands of phenol oxidase activity (derived from ebony) displayed similar responses in all cases.

The non-enzymatic reactions were included in Table 10 to show that excess dopa also inhibits the non-enzymatic

hydroxylation of tyrosine to dopa. The appropriate subtractions were made to derive the enzymatic values.

It appears, therefore, that dopa exerts two diverse effects upon the hydroxylation reaction catalyzed by banded phenol oxidase, the particular effect observed dependent upon the presence of $DMPH_4$. In the presence of the pteridine, dopa has an inhibitory effect upon the "tyrosine hydroxylase" activity; in the absence of $DMPH_4$, this same concentration of dopa stimulates the "hydroxylase" in comparison with the case where both compounds are absent.

Phenylalanine hydroxylase activity in heavy band phenol oxidase Heavy band phenol oxidase was assayed for its ability to catalyze the hydroxylation of phenylalanine to tyrosine. It will be recalled from the Introduction that there is evidence that tyrosine hydroxylase is capable of catalyzing this reaction.

50µl of enzyme were incubated with phenylalanine, at a final concentration of 0.19 mM in the presence and absence of $DMPH_4$ (at 0.4 mM when present). $DMPH_4$ is essential for this reaction to occur. The reaction conditions were those described in Materials and Methods, section 5 and tyrosine was assayed by the paper chromatography technique (see Materials and Methods, section 4B). Pteridine stimulatable activity was not observed in this assay.

It will also be recalled from the section on phenylalanine hydroxylase that this enzyme is extremely unstable, and since the sucrose gradients were run for 16 hours, these negative data do not lead one to an ineluctable conclusion concerning the possible association of phenylalanine hydroxylase with banded phenol oxidase.

The nature of the $DMPH_4$ inhibition of the dopa oxidase reaction and the stimulation of "tyrosine hydroxylase" activity

It is obvious from all of the above experiments that $DMPH_4$ plays a crucial role in the in vitro metabolism of tyrosine and dopa. It is the purpose of this section to relate information concerning the nature of these processes. An understanding of these phenomena should be helpful in the acquisition of a knowledge of the nature of the enzyme(s) involved in dopa synthesis.

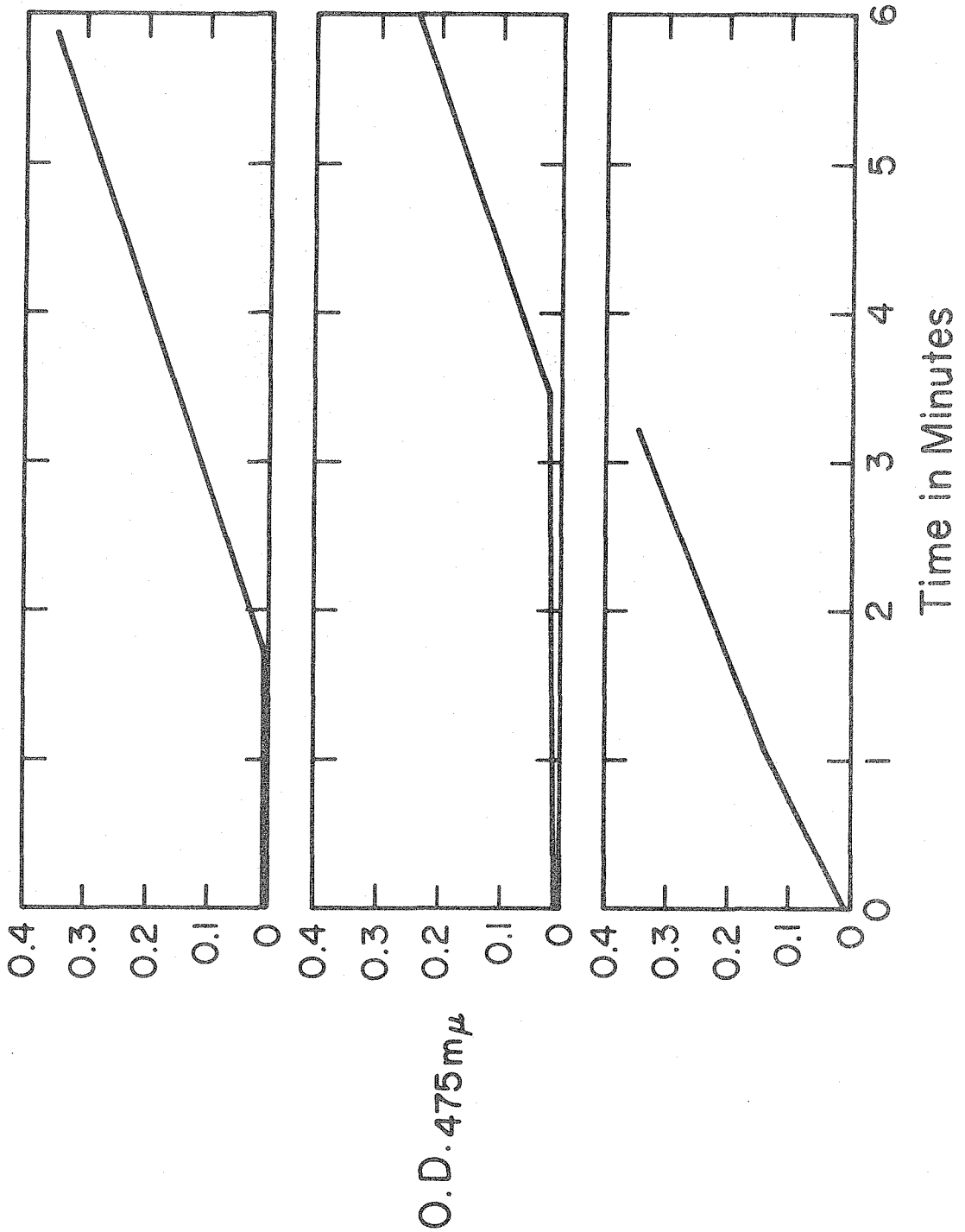
Inhibition of dopa oxidase activity It will be recalled from the data presented in Table 4 that if a high enough concentration of $DMPH_4$ is included in the hydroxylation assays, the accumulation of origin material is severly inhibited. This effect was explored further by monitoring the dopa oxidase activity via the change in optical density at 475mμ, the wavelength at which dopachrome (a product of the dopa oxidase catalyzed reaction) maximally absorbs.

The results from such an experiment are graphically depicted in Figure 18. $DMPH_4$ produces two effects upon the dopa oxidase reaction (when dopa is the substrate): creation of a lag period before any product is detected - in the absence of pteridine, the reaction begins immediately; secondly, the rate of the reaction, once it does begin, is diminished in comparison to the no-addition case. The duration of the lag is also dependent upon the concentration of $DMPH_4$. $DMPH_4$ does appear to serve at least one positive function for the reaction - the linear phase of the reaction is lengthened in comparison with the reaction sans $DMPH_4$.

It was found that if a high enough concentration of pteridine was utilized at a fixed concentration of enzyme, the lag period could be extended indefinitely. In one particular experiment, after a lag, the cuvette was removed from the instrument and it was noted that the top few millimeters of liquid had begun to give the characteristic darkening reaction indicative of dopa oxidase activity. Of course, the top of the column of liquid is richest in oxygen, which suggests that the pteridine must be in its reduced form to be effective in blocking the dopa oxidase reaction. Pteridines are readily air oxidizable.

The same pattern of results is achieved when banded phenol oxidase is utilized as the source of enzyme.

Figure 18. The effect of pteridine upon the kinetics of the dopa oxidase reaction. A crude enzyme preparation (in a volume of 25 µl) was reacted with 0.5 ml of 2 mM dopa in 0.1 M phosphate, pH 6.1 and either of two concentrations of pteridine or water alone. Pteridine was put into solution with glass distilled water prior to the assay. Weighings and measurements were performed as quickly as possible. The time course of the dopa oxidase reaction was followed by monitoring the change in optical density at 475 mµ in a Cary 15 spectrophotometer. The curves shown here represent tracings derived from this monitoring. Curve a represents the reaction carried out in the presence of 0.08 mM pteridine, curve b in 0.16 mM pteridine and curve c in the absence of the factor.


0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
Time in Minutes
O.D. 475mμ

Comparison of the effect of $DMPH_4$ and ascorbic acid on the hydroxylation of tyrosine and the synthesis of origin material The above section indicated that $DMPH_4$ may serve to block the manifestation of dopa oxidase activity (as assayed for by the change in optical density at 475mμ) via a reducing agent phenomenon, that is, by reducing dopaquinone back to dopa, thereby preventing accumulation of dopachrome. It will be recalled from the Introduction that this may be the modus operandi of ascorbate and DPNH in stimulation of the "tyrosine hydroxylase" reaction of tyrosinase. $DMPH_4$ is a reductant, for it serves as a hydrogen donor to reduce the unincorporated oxygen atom in the classical and well documented phenylalanine and tyrosine hydroxylase systems. Therefore, $DMPH_4$ was compared with ascorbic acid, a traditional reducing agent, with respect to their abilities to participate in the "tyrosine hydroxylase" reaction and the inhibition of dopa oxidase activity.

A. The comparative effect of ascorbate and $DMPH_4$ upon the hydroxylation reaction

The standard hydroxylation assay, utilizing a crude enzyme preparation, was performed at a series of concentrations of pteridine and ascorbate and the results from such an experiment are displayed in Table 11.

With respect to dopa accumulation, $DMPH_4$ and ascorbic acid demonstrate similar properties: on a molar basis, both factors are approximately equivalent in their abilities to stimulate this reaction. However, ascorbate, mole per mole, is a much better inhibitor of dopa oxidase activity than is the pteridine. Ascorbate, at a final concentration of 0.115 mM is more effective in this respect (over the 35 minute interval) than is $DMPH_4$ at a final concentration of 0.92 mM.

Additionally, ascorbate at these concentrations inhibits the synthesis of peak I material, whereas $DMPH_4$, as noted previously and in this Table, is a potent stimulant of this reaction. In a relatively low concentration range, the accrual of peak I material is greater the higher the concentration of pteridine; however, when this factor is present at a concentration approximately an order of magnitude higher, the reaction is severely inhibited.

Another salient feature of Table 11 resides in columns 9 and 10 where both factors are present together in the assay. The accumulation of dopa is enhanced over that achieved when only one of the factors is present at the concentration used in the mixture. It is of interest to note that the non-enzymatic system duplicates this effect. Also, the synthesis of origin material is reduced when compared to that

Table 11

The Relative Effects of $DMPH_4$ and Ascorbic Acid on the Tyrosine Hydroxylation Reaction Catalyzed by a Crude Enzyme Preparation

product* measured in nmoles	0.9 mM $DMPH_4$	0.9 mM asc*	0.46 mM $DMPH_4$	0.46 mM asc	0.23 mM $DMPH_4$	0.23 mM asc
origin material	1.14	0.13	1.53	0.13	2.1	0.15
peak I material	0.77	0	0.83	0	0.46	0
dopa	11.9	8.4	8.9	7.8	8.0	6.5

* The standard hydroxylation assay (see Materials and Methods, section 5) was carried out in the presence of varying concentrations of pteridine and ascorbate. Both factors were made up at a concentration of 16 mM in 0.1M phosphate, pH 6.7 and diluted in a phosphate solvent buffered at pH 6.1. These steps were carried out immediately prior to the assay. Tyrosine was present at a final concentration of 0.46 mM.

** Ascorbic acid is abbreviated asc.

Table 11 (continued)

product measured in nmoles	0.12 mM $DMPH_4$	0.12 mM asc	0.46 mM $DMPH_4$ + 0.46 mM asc	0.23 mM $DMPH_4$ + 0.23 mM asc	no additions
origin material	2.39	0.24	0.14	0.3	2.40
peak I material	0.46	0.04	0	0.53	0.5
dopa	6.77	6.15	11.0	9.5	4.4

derived when the same concentration of $DMPH_4$ is used alone.

Although both factors appear to enhance the "tyrosine hydroxylase" reaction, they exert diverse effects upon the systems responsible for the synthesis of origin material and peak I material.

Qualitatively similar results were arrived at when both heavy and light band phenol oxidase were utilized as enzyme sources.

B. Relative effects of $DMPH_4$ and ascorbic acid upon the kinetics of the over-all tyrosinase reaction

The kinetics of the tyrosinase reaction in the presence of the two factors were determined by monitoring the change in optical density at 475mμ. This technique is a convenient one, however, the salient features of the first reaction, the hydroxylation of tyrosine, go undetected.

The original plan behind the experiments was to compare the relative effects of pteridine and ascorbate on the reaction using tyrosine and dopa as substrates. A value for relative inhibition of the dopa oxidase phase of the reaction was to be determined, as reflected by the duration of the lag period preceding dopachrome formation, and then compared to the lag achieved when tyrosine was the substrate. The assumption was that when "equi-equivalent" quantities of $DMPH_4$ and ascorbate, as determined by the reaction with dopa, were em-

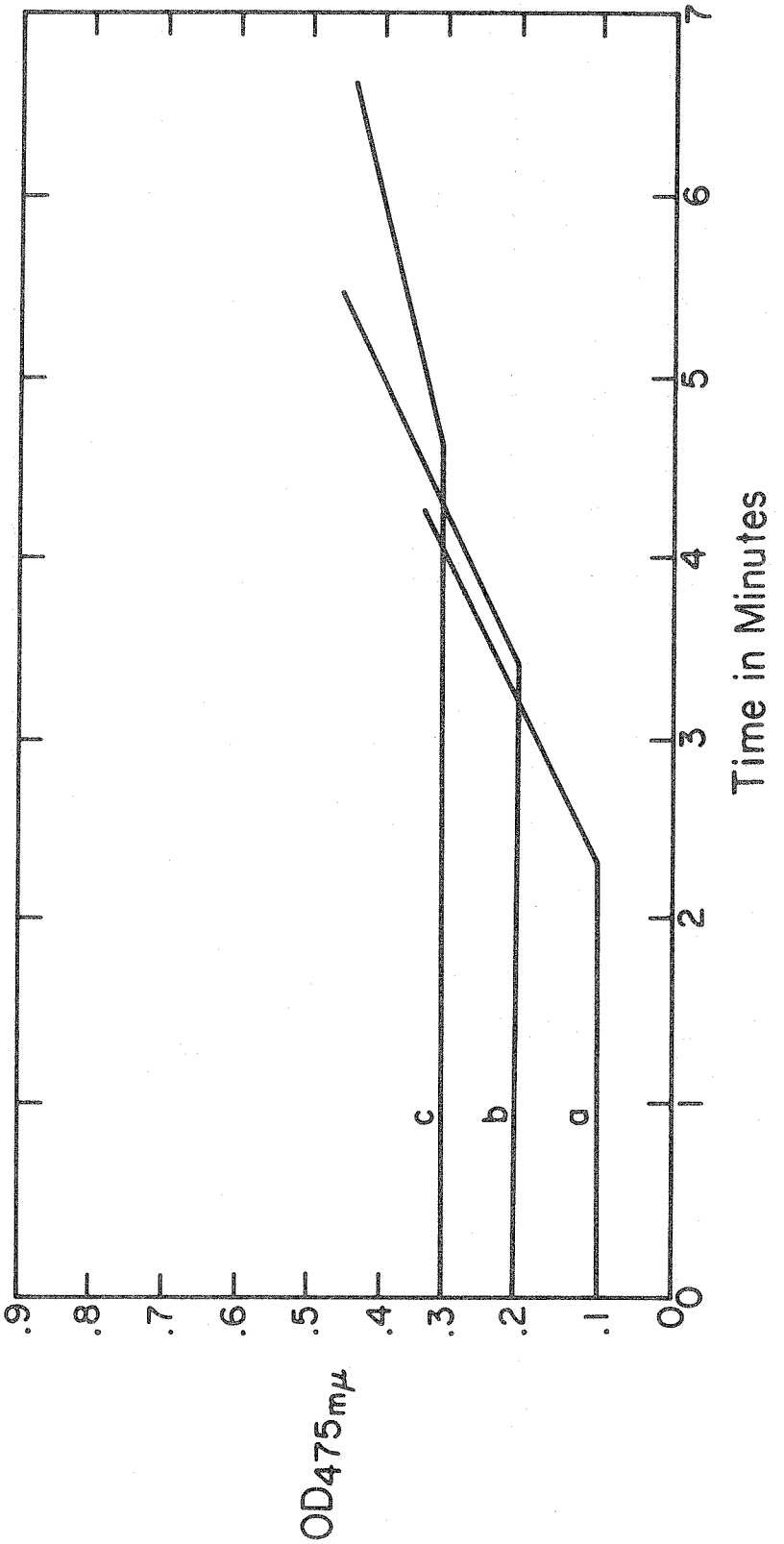
ployed in the tyrosine reaction, if $DMPH_4$ were stimulating the hydroxylation reaction beyond what the ascorbate was capable of, this lag period would be shortened because of the additional dopa provided for by the stimulation. Unfortunately, the results from many such experiments turned out to be extremely scattered and, therefore, of limited value.

Nevertheless, some information concerning the kinetics of the over-all tyrosinase reaction was accrued and is summarized in Figure 19. In the situation where $DMPH_4$ and ascorbate (curves a and b) are present at final concentrations of 0.08 and 0.04 mM respectively, the lag periods before onset of dopachrome formation are shortened in comparison with the case where neither factor was present. Additionally, the slopes of the linear portions of the curves derived in the presence of the factors are equal and are also greater than that observed in the no-addition case.

On occasion, the curves clearly demonstrated biphasic regions of activity, the second slope being much greater than the first, both in the presence of the factors and in their absence. The reason for this particular inconsistency in results is unknown.

In the case described in Figure 19, the equation, 1 mole of ascorbate = 2 moles of $DMPH_4$ with regards to ability to

Figure 19. The effects of pteridine and ascorbic acid upon the kinetics of the overall tyrosinase reaction. The conditions employed here were similar to those used in Figure 18, except that tyrosine (at 2 mM) was used instead of dopa as the substrate. Also, the solutions containing pteridine and ascorbate were made up in advance and quickly frozen in liquid nitrogen and stored at -80°C. Curve a represents the kinetics of the reaction occurring in the presence of 0.08 mM pteridine, curve b in 0.04 mM ascorbate and curve c in the absence of either factor.


OD475mμ
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0
1
2
3
4
5
6
7
Time in Minutes
a
b
c

arrest the dopa oxidase reaction (with dopa as substrate) was derived. In this Figure, where $DMPH_4$ is present at twice the molar level of ascorbate, the duration of the lag is less than that for the ascorbate case. Hence, even though the adjustment in concentration of the two factors results in equal lag periods when dopa is the substrate, when tyrosine is used, the $DMPH_4$ reaction appears to begin more rapidly, suggesting a stimulation of the hydroxylation reaction beyond that occuring when an "equi-equivalent" level of ascorbate is used. As mentioned above, this type of experiment was extremely difficult to reproduce, probably because of the unstable nature of the two factors.

Hence, it is impossible to draw any firm conclusions from these endeavors, other than $DMPH_4$'s effect on the abrogation of the lag period preceding onset of dopachrome formation is similar to that observed when ascorbic acid is employed in its stead.

C. The comparative effects of ascorbic acid and $DMPH_4$ upon the reactions catalyzed by mushroom tyrosinase

In order to investigate further the modes of action of $DMPH_4$ and ascorbate upon the tyrosinase system, standard hydroxylation assays were carried out using mushroom tyrosinase as the source of enzyme. There has been no in-

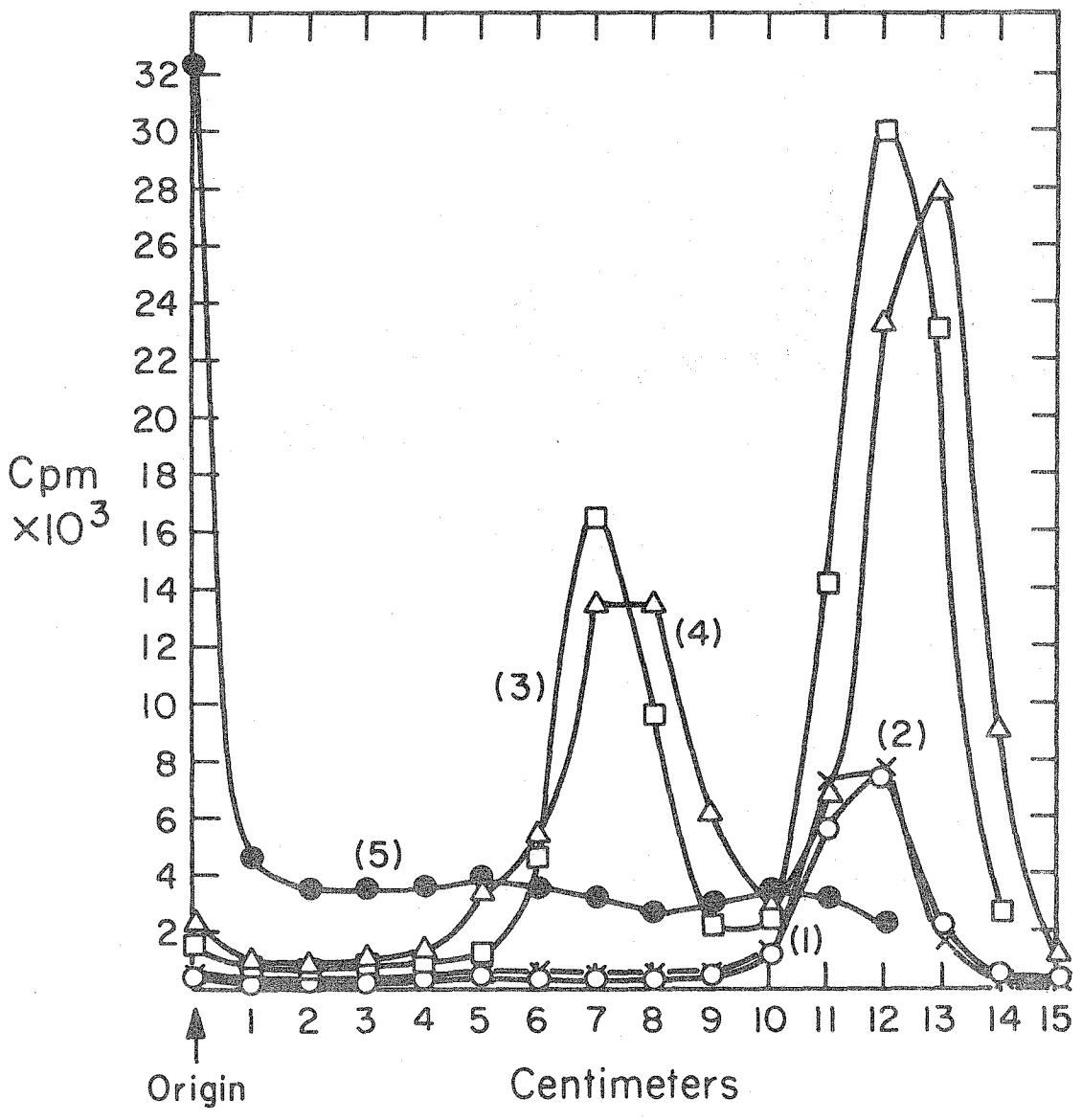
formation reported concerning the effect of pteridine on this enzyme. The results from such an experiment are displayed in Figure 20. A normal reaction utilizing banded ebony phenol oxidase was run along for comparative purposes.

It is obvious from these data that the accrual of dopa in the mushroom system is tremendously enhanced in the presence of both $DMPH_4$ and ascorbate (curves 4 and 5) in comparison with the reaction sans either factor (curve 6). Furthermore, the dopa oxidase function of the mushroom preparation is severely inhibited by both factors at these concentrations (9.15 mM for $DMPH_4$ and 14.3 mM for ascorbate). Both of these effects are reproduced in the Drosophila enzyme system.

There is one glaring difference between the reactions catalyzed by these two enzyme systems, namely, the appearance of another metabolite in the chromatograms derived from the mushroom enzyme reaction. The appearance of this compound is dependent upon the presence of either pteridine or ascorbate. Its position on the chromatogram is fairly close to that where Drosophila peak I material migrates when it is present, although it may move a bit faster than does peak I material. There is no hint of this compound's presence in the Drosophila chromatograms. The concentrations of factors used in these particular assays are large enough to block the appearance of Drosophila peak I material when the enzyme

Figure 20. The relative effects of pteridine and ascorbate upon the reaction catalyzed by mushroom tyrosinase. Mushroom tyrosinase (25 µl of 0.25 mg/ml 0.1 M phosphate, pH 6.1) was utilized as a source of enzyme for the standard assay (see Materials and Methods, section 5). Tyrosine was present at a final concentration of 0.11 mM, pteridine (when present) at 9.1 mM and ascorbate (when present) at 14.3 mM. A similar set of reactions was carried out using 25 µl of Drosophila heavy band tyrosinase.

Curve 1 represents Drosophila enzyme plus pteridine, curve 2 Drosophila enzyme plus ascorbate, curve 3 mushroom enzyme plus pteridine, curve 4 mushroom enzyme plus ascorbate and curve 5 mushroom enzyme alone.


Cpm
×10³
32
30
28
26
24
22
20
18
16
14
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8
6
4
2
(1)
(2)
(3)
(4)
(5)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Origin
Centimeters

preparation possesses the capability to synthesize it. It will also be recalled that relatively low concentrations of ascorbate are completely inhibitory to this reaction.

The mushroom tyrosinase system responds to $DMPH_4$ and ascorbate in the same manner as does the Drosophila system with regard to inhibition of dopa oxidase activity and the accrual of dopa. However, these two factors enhance the accumulation of another metabolite which is not seen in the Drosophila reactions under these conditions, but they do appear to act similarly in the mushroom system in this respect.

DISCUSSION

1. The conversion of phenylalanine to tyrosine

The data presented demonstrate that Drosophila possesses a system which is capable of converting phenylalanine to tyrosine *in vivo* and that the enzyme(s) responsible is readily extractable.

That tyrosine is a product of an incubation of phenylalanine(C^{14}) both *in vivo* and *in vitro* is substantiated by carrying out paper chromatography of extracts in various solvent systems and detecting a peak of activity corresponding to the appropriate R_f of tyrosine in each case. Further evidence that tyrosine is derived from phenylalanine *in vitro* comes from the fact that the product of the reaction between nitroso-napthol and the assay material displays fluorescent spectral characteristics identical to those derived when tyrosine is used in this reaction.

The phenylalanine hydroxylase systems which have been studied in other organisms display an absolute dependency on tetrahydropteridine for activity. Drosophila extracts treated with Sephadex G-25 to remove low molecular weight compounds are totally lacking in ability to catalyze the conversion of phenylalanine to tyrosine. When the included fraction from

the G-25 column is added back, partial restoration of activity results. Hence, Drosophila requires at least two components to catalyze the synthesis of tyrosine-a protein and a low molecular weight substance.

The synthetic pteridine, 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine, when added to the excluded fraction derived from G-25 treatment, results in a very active conversion of phenylalanine to tyrosine in the incubation mixtures. This strongly suggests that the enzyme responsible for the catalysis is, indeed, a hydroxylase.

The fact that all of the common biological reductants tested (NADH, TPNH,ascorbate and tetrahydrofolate) are completely incapable of replacing the pteridine in this system strongly indicates that the pteridine plays a specific role in the hydroxylation reaction and is not merely serving as a non-specific hydrogen donor for the reduction of the unincorporated oxygen atom. This specificity is like that seen in the rat liver system, where Kaufman has postulated that the pteridine actually serves as a fully oxygenated intermediate (a "tetrahydropterin hydroperoxide") in the hydroxylation reaction (29).

It should be noted that attempts were made to isolate the endogenous cofactor from Drosophila larval extracts by a variety of techniques, however, substantial activity could never be recovered.

The exact physiological significance of the hydroxylase system is not clear. Under normal laboratory rearing conditions (and presumably in nature), the animals are able to obtain tyrosine from their diet. It has been mentioned previously that tyrosine is not an essential amino acid for Drosophila, but that phenylalanine is (25).

The data of Chen and Hanimann show that the level of free phenylalanine in the whole animal decreases during the larval stages (from 1 μmole/g to 0.13 μmole/g), while the levels of free tyrosine and tyrosine-O-phosphate, which is observed in late third instar larvae, is approximately 5 μmoles/g or 10 nanomoles/larvae (113). The actual amount of tyrosine that is synthesized during the larval stages of development is probably greater than this because, as has been suggested by Mitchell (97) and Seligman, et al. (15), a tyrosine rich storage protein may exist. It is likely the tyrosine is immediately incorporated into the storage protein and would thusly be undetected in Chen and Hanimann's procedures. It will be recalled from the injection experiments reported above that larvae of this age are capable of converting 0.12 nanomoles of phenylalanine to tyrosine in a 20 minute incubation. However, there was no hint that the larvae were saturated and, doubtless, the animal is capable, if called upon by metabolic needs, to furnish an ample supply of tyrosine for cuticle formation.

A study of how well the animals develop cuticle and display pigmentation when reared on a tyrosine free diet would aid in evaluating the importance of phenylalanine hydroxylase in providing tyrosine for sclerotization and melanization. It would also be of interest to assay the levels of enzyme under these conditions. In the absence of tyrosine that is normally derived from the diet, the levels of hydroxylase may increase in analogy to microbial biosynthetic systems.

It is of interest that the level of phenylalanine hydroxylase is a function of the stage of development. The peak of activity occurs in late larval life, the time at which large quantities of tyrosine are needed for cuticle synthesis, suggesting that the hydroxylase does play a critical role in this pathway. As soon as the puparium is formed, the level of hydroxylase drops to less than 1 nanomole of tyrosine synthesized/mg of protein, the level which is maintained throughout pupal life. The amount of free tyrosine in the animal remains at a relatively low level during this period (113). Presumably, the tyrosine that is need is derived from turnover of pre-existing proteins. One might conjecture that the hydroxylase may reappear near the end of pupation, for at this time, the animal undergoes extensive pigmentation, a process which requires tyrosine. As noted in the Results, there may be a slight amount of activity present just prior

to eclosion. It is also possible that this new peak of activity is sharp and that it was merely missed in these experiments.

Chen and Hanimann's data show that day old larvae contain a significant amount of tyrosine. Figure 10 shows that 24 hour larvae do not possess detectable levels of hydroxylase activity. The tyrosine that is observed at this stage may be derived from a previous period of activity that was not detected in this experiment.

The various eye color mutants, which are apparently defective in pteridine metabolism, demonstrated relatively equivalent levels of total hydroxylation capacities in comparison to the wild type (Oregon R). The exact positions of the blocks in the pteridine pathway are unknown so that an active pteridine (or derivative) is likely synthesized, thusly allowing for manifestation of hydroxylase activity.

The enzymological characteristics of crude Drosophila phenylalanine hydroxylase are relatively straightforward. The activity of the enzyme responds linearly (up to the point of saturation) to substrate, cofactor and enzyme concentration. The kinetics of the reaction carried out in excess substrate and cofactor are also linear.

It had been hoped to be able to study the nature of the phenylalanine hydroxylase system, in particular, the means by which its activity was regulated. The potential for the existence of interesting regulatory mechanisms appears to

be large. As mentioned in the Introduction, the synthesis and function of the first enzyme in a biosynthetic pathway is often subject to tight regulation. This particular system possesses the additional possibility of interaction with the pteridine biosynthetic pathway and also the enzyme responsible for the TPNH dependent regeneration of the pteridine.

In order to elucidate these mechanisms, relatively pure enzyme is required. Attempts at purification of the hydroxylase proved to be fruitless. The unstable nature of the enzyme, as described in Table 3, coupled with the fact that the yield of activity in crude extracts was low in comparison to the potential expressed *in vivo*, certainly had a large effect on the outcome of these endeavors.

Drosophila phenylalanine hydroxylase behaves as a large molecule (at least a molecular weight of 500,000 daltons) as evidenced by its exclusion on Bio-Gel 0.5. It is possible the enzyme exists as an aggregate under these conditions of low salt (0.1 M buffer). High salt, urea, and detergent, conditions conducive toward dissociation, are deleterious to the activity of the enzyme and were not utilized as a consequence. There was no indication in these experiments that the enzyme exists in multiple forms.

The nature of the enzyme(s) responsible for the substrate independent accrual of tyrosine is unknown. If it is,

indeed, a proteolytic activity, it would not be possible to draw any firm conclusions about it since the substrate (other proteins) is so variable in these assays. Another consideration is that the tyrosine rich protein referred to above may be serving as a substrate for some sort of hydrolytic activity in these assays.

2. Conversion of tyrosine to dopa

The data presented clearly demonstrate that crude Drosophila extracts possess the ability to convert tyrosine to dopa, a reaction which is stimulated by tetrahydropteridine. The co-chromatography of a distinct peak of C^{14} (when C^{14}-tyrosine is incubated with the extracts) with H^3-dopa demonstrates that the material in the C^{14} peak is dopa.

The central consideration of this process is the identity of the enzyme(s) involved in synthesizing dopa and the attendant controls placed upon it. As mentioned in the Introduction, tyrosinase is quite capable of catalyzing the ortho-hydroxylation of tyrosine to yield dopa, however, since all tyrosinases studied to date contain very active dopa oxidase functions in vitro , the dopa is rapidly converted to melanin. Of course, if tyrosinase were the sole catalytic activity involved in supplying the animal with dopa, means would have to be provided to shunt some of the dopa to the sclerotization pathway.

The kinetics of appearance of dopa and origin material in crude extracts suggest that dopa arises from at least two separate pathways (see Figure 15). The rate of appearance of origin material (utilization of dopa) during the 15-25 minute period of the reaction was higher than the rate of decrease of free dopa during this time interval. If dopa were arising

from only one catalytic source, one would expect to see a decrease in the rate of accrual as the dopa was being consumed by dopa oxidase activity. This experiment was repeated and similar results were obtained. In this case, the rate of accrual of dopa was linear over an 18 minute interval while the rate of accumulation of origin material displayed a sigmoidal curve similar to the one depicted in Figure 15 except that there was a distinct increase in rate during the 12-18 minute period over the initial 12 minute period, again indicating that dopa arises from at least 2 pathways. Of course, it is likely that the dopa from both pathways is used *in vitro* by dopa oxidase and this could account for the large decrease in rate of dopa accumulation during the 25-40 minute period.

When the crude system was subjected to ammonium sulfate fractionation, the 3 activities responsible for synthesis of origin material, peak I and dopa behaved similarly. The material which precipitated in 40% $(NH_4)_2SO_4$ contained catalytic activities responsible for the synthesis of all 3 metabolites, but on mixing this fraction with the 40-60% fraction, all 3 activities were enhanced to about the same extent. This stimulation upon mixing of the 2 fractions bears a strong resemblance to the tyrosinase system originally described by Mitchell, *et al.* (88) and recently in more detail by Seybold, *et al.* (110). In this scheme, 2 ammonium

sulfate fractions are derived: a 0-35% fraction which contains S,P and Pre-S components and a 36-56% fraction which contains the A components (A_1, A_2, A_3) and the S activator. Each fraction is inactive when used alone, but upon combination, tyrosinase activity is derived. It is likely in the experiment described in Table 7 that the 0-40% fraction was contaminated with some of the 40-60% material. Extensive washing of the pellets as a precaution against such contamination was not carried out.

It is of considerable interest to note that in one experiment where more total activity was present than the one shown in Table 7 the dopa oxidase function was activated 15 fold while the "tyrosine hydroxylase" was stimulated only 3 fold upon combination of the 2 fractions. This situation is one example of a phenomenon noted in a number of other experiments where it appears that dopa oxidase and "tyrosine hydroxylase" are activated in different fashions.

Another example of this phenomenon resides in the experiments where "tyrosine hydroxylase" and dopa oxidase activities were measured as functions of the age of the animal. The ratio of the 2 activities was dependent upon the concentration of the extract used, the more concentrated having a lower "tyrosine hydroxylase" to dopa oxidase ratio than the dilute one. It should be pointed out that the level of"tyrosine

hydroxylase" activity was increased 20 fold when the concentration of the extract was increased by a factor of 1.8, indicating that "tyrosine hydroxylase" also is dependent upon a critical concentration of components. Dopa oxidase activity was enhanced by a factor of 60 under these conditions. Additionally, it was noted that the preparations underwent an "aging" process whereby dopa oxidase became more active than "tyrosine hydroxylase". These phenomena reflect the activation process which tyrosinase undergoes, a process which is dependent upon the concentration of the extract (111). In these cases, especially with the younger animals, dopa oxidase activity was more dependent upon concentration of components. It is likely that one or more of the components was present in limiting concentrations, so that the activation process was relatively ineffecient, requiring a higher initial concentration of extract and more time to acheive full activity. This same reasoning applies to the differential activations of "tyrosine hydroxylase" and dopa oxidase in the ammonium sulfate experiments. Presumably, in the case where dopa oxidase was activated so much more than the "tyrosine hydroxylase", the fractions were richer in components necessary for dopa oxidase activity than in the experiment delineated in Table 7.

Meltzer has suggested that the relative concentration of the A components of tyrosinase is important in determin-

ing the enzyme's substrate specificity (96). It has been demonstrated that the A_1 component , which gives rise to tyrosinase with both mono and diphenol oxidase activities, is present throughout the early stages of larval development and has its peak of activity around puparium formation (97). The A_2 and A_3 components, which give rise to tyrosinase possessing only diphenol oxidase activity, are present at much lower levels than A_1 during the earlier stages of larval development. This situation is consistent with the increased "tyrosine hydroxylase" to dopa oxidase ratios observed in early third instar larvae in comparison with older animals. The facts that enzyme preparations "age" (activate), the level of dopa synthesized is dependent upon the concentration of the extract in a non-linear fashion and the enzyme(s) responsible for the synthesis of dopa responds to ammonium sulfate fractionation in much the same way as does tyrosinase, suggest that a percentage of the observed "tyrosine hydroxylase" activity is due to the action of the tyrosinase complex.

The data derived from equilibrium sucrose gradient centrifugation of activated tyrosinase demonstrate that banded tyrosinase, replete with dopa oxidase activity, possesses the ability to catalyze a $DMPH_4$ stimulated hydroxylation of tyrosine in the same manner as do crude extracts. However,

the two bands of activity derived from ebony display differences in their abilities to utilize tyrosine and dopa as substrates. The heavy banded material is less able than the light banded to catalyze the overall tyrosine to melanin reaction although it is well equipped to hydroxylate tyrosine and catalyze the dopa to melanin reactions. Hence, this particular species of tyrosinase is well adapted to meet the exigencies of a system that provides dopa for sclerotization, for here the newly synthesized dopa has the opportunity to escape from further oxidations. It should be noted that the heavy band does oxidize some of the dopa to melanin, but not as effeciently as does the light band. Of course, it is not known if the species of tyrosinase derived from the gradients reflect the state of the enzyme in vivo. Nevertheless, it is likely that these two species of tyrosinase possess different proportions of components in analogy to the situation discussed above.

The banded tyrosinase complex in vitro responds to metabolic regulation in an interesting fashion. When excess dopa is included in the standard assay (C^{14}-tyrosine as the substrate), two phenomena are noted: in the presence of $DMPH_4$, the "tyrosine hydroxylase" activity is inhibited by 46%; secondly, in the absence of the pteridine, dopa stimulates "tyrosine hydroxylase" activity by a factor of 6 over the

case where neither compound is present. It is of interest to note that dopa inhibition occurs only in the presence of $DMPH_4$, the situation where the hydroxylase function is at its most active. Dopa appears to be acting as a classical end product inhibitor in this assay. Pomerantz has reported that dopa competes with tyrosine in the hydroxylation reaction catalyzed by tyrosinase from mouse melanosomes (67,79).

The means by which dopa stimulates the synthesis of more dopa in the absence of pteridine is not clear. It has been known for a number of years that small amounts of dopa stimulate the hydroxylation reaction, probably by providing for the reduction of the extra oxygen atom to water , and in the process is oxidized to dopaquinone. $DMPH_4$, whether it is acting as a cofactor actually participating in the enzymatic reaction or as a "non-specific" reducing agent (see subsequent discussion), produces the same effect as would dopa if it were serving as an electron donor. $DMPH_4$ was present at 4 times the concentration of dopa, which itself was present at a 20 fold excess in comparison with the substrate tyrosine. This concentration of dopa should be equivalent to that of $DMPH_4$ in that they are both present in saturating quantities (as far as providing hydrogen for reduction), however, the reaction with dopa is one-half as active as the one with the pteridine. Dopa may not be present at saturating levels

because some of it is being consumed in an uncoupled manner in the melanization process. The rate of dopa oxidation is greater than the rate of tyrosine oxidation in the mushroom tyrosinase system(73). Dopa may also be a competitive inhibitor of tyrosine in this reaction, so that it is difficult to interpret the role of dopa in this particular case.

When enough enzyme was used, it was noted that a 20 fold molar excess of C^{12}-dopa inhibited the accrual of origin material (with C^{14}-tyrosine as substrate) by 65%. If tyrosine and dopa shared active sites, one would expect the inhibition to have been much larger since dopa is in such great excess.

These results are consistent with ones derived by Kim and Tchen using tyrosinase isolated from goldfish (106). When excess cold dopa was included in their hydroxylation assays, they noted very little inhibition of melanin formation, which led them to postulate that free dopa is not an intermediate in the reaction, but it remains enzyme bound and is, therefore, protected from exchange with free dopa.

The fact that there is some inhibition shows that, if dopa were an enzyme bound intermediate, the binding is not that tight and some dopa may be able to escape from subsequent oxidations. Alternately, this partial inhibition could reflect the existence of two distinct dopa oxidase active sites (each having its own hydroxylase activity associated with it), one

of which protects the dopa from exchanging with free dopa because of the nature of the interaction between the particular hydroxylase site with the dopa oxidase site. The other activity could represent one in which the dopa were more likely to escape.

The role of $DMPH_4$

A major feature of all of the results presented is that $DMPH_4$ exerts two very pronounced effects upon the reactions: prevention of melanin formation and stimulation of dopa accumulation. $DMPH_4$ is a known cofactor for mammalian tyrosine hydroxylase (89,90). Obviously, if the role of $DMPH_4$ in these reactions is defined, a better understanding of the nature of the enzyme(s) involved in synthesizing dopa can be attained.

$DMPH_4$ may be acting in one of two ways (or both) in these reactions: it could be serving as a specific cofactor for the hydroxylation reaction in that it interacts with the enzyme and participates in the actual reaction and is itself oxidized in the process,or it could be serving in a nonspecific manner to provide for the reduction of the unincorporated oxygen atom to water. This latter mechanism may be indirect in that $DMPH_4$ reduces dopaquinone which is generated upon release of 2 hydrogen atoms to reduce the oxygen. Since reduced pteridine does inhibit the accumulation of

melanin (see Figure 18), it is likely that it does function in the latter mode, however, the extent of its involvement in the former situation is not clear. It is possible that reduced pteridine actually interacts with dopa oxidase in an inhibitory manner.

To evaluate properly the role of $DMPH_4$ in these reactions, the mechanisms of action of tyrosine hydroxylase and tyrosinase have to be delineated. Since the tyrosine hydroxylase mechanism has not been carefully studied to date, use is made of the work done on the phenylalanine hydroxylase system, which likely functions in the same manner as does the tyrosine hydroxylase. Kaufman has presented kinetic data which substantiate this hypothesis (29).

Mager and Berends have postulated that tetrahydropteridines can react with oxygen non-enzymatically to form transient hydroperoxide derivatives ($DMPH_3$-OOH) (102). The hydroperoxide can participate in three reactions:

1.) $DMPH_3\text{-}OOH + RH \longrightarrow R\text{-}OH + DMPH_3\text{-}OH$

2.) $DMPH_3\text{-}OOH + DMPH_4 \longrightarrow 2\ DMPH_3\text{-}OH$

3.) $DMPH_3\text{-}OOH + H_2O \longrightarrow DMPH_3\text{-}OH + H_2O_2$.

The first reaction results in the introduction of a hydroxyl group into an appropriate molecule (such as, phenylalanine and tyrosine). The hydroperoxide derivative also undergoes reactions with more $DMPH_4$ (reaction 2) and water

(reaction 3) to yield the hydroxy-pteridine derivative, processes which effectively reduce the concentration of "active" $DMPH_4$. Hydroxy - pteridine splits off water to yield $DMPH_2$ which can be reduced back to $DMPH_4$ via a TPNH dependent reaction.

The only problem to the above scheme of reactions is that the proof of the existence of $DMPH_3$-OOH is only inferrential. If such a compound were proven to exist in the situation where Drosophila enzyme is incubated with tyrosine, a large step would be taken in understanding the means by which dopa arises. Since this compound reacts readily with more $DMPH_4$ and water, isolation of such a compound would be extremely difficult. A system of spectroscopic analyses which can follow the kinetics of very rapid reactions could be set up. First of all, the rate of accumulation of the hydroperoxide-pteridine which occurs non-enzymatically could be compared to the rate derived in the presence of enzyme to determine if the enzyme is involved in the initial step. The rates of disappearance of the hydroperoxide in the presence and absence of substrate would also yield useful information, for if this rate were larger in the presence of substrate, this would be a strong indication that the reaction proceeded as described above.

Kaufman has demonstrated in the phenylalanine hydroxylase system that if either substrate or cofactor analogs are

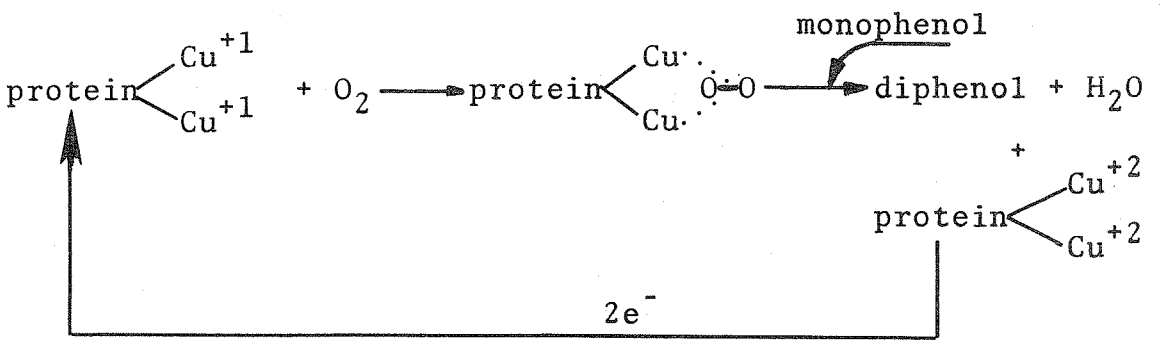
utilized, consumption of $DMPH_4$ becomes non-stoichiometric in relation to hydroxylated product, or, in other words, the two reactions become loosely coupled (38). When this situation exists, oxygen becomes the hydrogen acceptor (derived from oxidation of $DMPH_4$ to $DMPH_2$) and hydrogen peroxide is produced. A scheme for this process is depicted here:

$$\text{enzyme } (DMPH_2,\ O_2^{=},\ 2H^+,\ RH) \begin{cases} \xrightarrow{a} \text{enzyme} + DMPH_2 + RH + H_2O_2 \\ \xrightarrow{b} \text{enzyme} + DMPH_2 + ROH + H_2O. \end{cases}$$

Whether or not reaction a or b occurs depends on the nature of the pteridine and the substrate (RH). This model does not distinguish between a $DMPH_3$-OOH or RH-OOH intermediate, but in either event, peroxide is released when the improper co-factor or substrate is used. Either the ratio of $DMPH_4$ consumed to product formed or the level of peroxide evolved can be used to assay these events. It would be of interest to use Drosophila enzyme in this sort of system, for information can be garnered concerning the mechanism of the hydroxylation reaction and also the involvement of pteridine in this process. If uncoupling is noted, this would suggest that $DMPH_4$ plays an active, specific role in the reaction.

The exact chemical mechanism by which tyrosinase operates has not been unequivocally defined, although some tenable theories have been presented. All models must accomodate the existence of a copper prosthetic group. A generalized scheme

for the tyrosinase reaction is depicted here (112):



The nature of the interaction between molecular oxygen and enzyme bound cuprous ion is unknown. It is possible that the oxygen is bound at the level of peroxide, analogous to the proposed mechanism for tyrosine hydroxylase. Another similarity that these two mechanisms share is the need for reductants. Classically, in the tyrosinase system, it was assumed that the electrons needed to reduce cupric back to cuprous came from the coupled oxidation of dopa to dopaquinone, hence, the need for catalytic amounts of dopa to initiate the hydroxylation reaction. Non-specific reducing agents, such as, ascorbate and DPNH, can fulfill this requirement (74,75). An additional fact of interest arises from Pomerantz's work on mouse tyrosinase whereby he demonstrated that $DMPH_4$ was more effective than ascorbate in relieving the lag period in the monophenolase reaction (79).

Even though both tyrosine hydroxylase and tyrosinase require reducing agents for activity, the two systems differ

in that tyrosine hydroxylase is very specific in its requirement for a hydrogen donor, whereas the tyrosinase system appears to be able to utilize any compound with an adequate reduction potential, at least *in vitro*. In either case, the end result is the same: two reduction equivalents are consumed to yield one molecule each of diphenol and water. Furthermore, the oxygen donor in both reactions is molecular oxygen.

When the relative effects of $DMPH_4$ and ascorbate on the "tyrosine hydroxylase" and dopa oxidase activities in crude *Drosophila* extracts were measured, it was found that both factors served to arrest melanin formation and also stimulate the synthesis of dopa, the latter effect to about the same degree. These effects were also achieved in like manner when banded tyrosinase was used. Ascorbate at a concentration of 0.12 mM allowed only a slight accrual of origin material, whereas $DMPH_4$ at 0.9 mM permitted a substantial synthesis of this substance during the 35 minute reaction period. It will be recalled from results derived in the colorimetric assays that, on a molar basis, ascorbate was about two times as effective as the pteridine in arresting the accumulation of melanin as measured by the duration of the lag before onset of color formation, and also, if the appropriate adjustments were made in concentrations,

the two reactions began at about the same time when tyrosine was the substrate. The reaction conditions in these two types of assays were different in that the concentration of substrate was higher in the latter assays and also the cuvette was open, thereby allowing for a higher concentration of oxygen which is a participant in both the hydroxylation reaction and the auto-oxidation of the factors. The tubes were capped in the C^{14} assay. In fact, when the quantity of dopachrome is calculated in the colorimetric assay, using a molar extinction coefficient of 3600 (108), the final level of product was 2-3 times greater in this assay than in the C^{14} assay.

When combinations of the two factors were used, the level of dopa accumulated was higher than when the same concentration of each was used singularly. This synergistic phenomenon bespeaks of the existence of two separate mechanisms for the synthesis of dopa, each factor participating in a separate reaction. However, if two such systems did exist independently, one would expect a purely additive effect. Although this was not the case, it is possible that there is a competition for substrate by the two pathways which results in one system operating sub-optimally. Another possibility is that one factor, having a more negative reduction potential than the other, serves to reduce the other factor, or else consume oxygen more effeciently, thereby allowing

for a more effective concentration of reductant. It is also of interest that the non-enzymatic hydroxylation of tyrosine displays this synergistic effect. This does not necessarily imply that only one chemical mechanism is operative: $DMPH_4$ could still be serving as a hydroperoxide donor as noted above.

Obviously, it would be of value to know the relative reduction potentials of $DMPH_4$ and ascorbate in order to evaluate properly the role of the pteridine in the reactions. Since both factors are extremely reactive, and especially so under the conditions employed in these assays, it was thought best to determine these relative values by measuring their abilities to arrest melanin formation in crude extracts. As noted above, the equation of 1 mole ascorbate = 2 moles of $DMPH_4$ was derived in these experiments. When tyrosine was used as the substrate and the length of the lag period measured, it was found that, even after the appropriate adjustment in concentrations were made, $DMPH_4$ decreased the lag, suggestive of a stimulation beyond what a non-specific reducing agent (ascorbate) could provide. Unfortunately, this was a very difficult experiment to reproduce, so that the validity of these data is somewhat dubious.

It is also of considerable interest that pteridine and ascorbate exert similar effects on mushroom tyrosinase. A

role for $DMPH_4$ has not been described for this enzyme, nor has its existence been reported in this organism, however, its reduction potential does allow it to serve as an electron donor in a non-specific sense.

The weight of the evidence presented above concerning the relative effects of pteridine and ascorbate on the hydroxylation of tyrosine suggests that these two factors serve the same function in the reaction. These data cannot distinguish between any differences in the modes of action of each, but only describe the end result of the process. It is possible that, when $DMPH_4$ is present, a "tyrosine hydroxylase" reaction takes place, with the pteridine-hydroperoxide intermediate serving as the oxygen donor for the hydroxylation, and that when ascorbate is used, a tyrosinase reaction is scored whereby the ascorbate functions in a less specific way than does the pteridine.

To settle the question unequivocally, more direct means of ascertaining the role of the factors must be utilized. As mentioned before, spectroscopic analyses of the reaction mixtures in an attempt to identify transient intermediates would be profitable. Equilibrium dialysis with labelled pteridine and Drosophila enzyme could be a useful tool in evaluating the role of $DMPH_4$. If selective binding were observed, this would be indicative of an intimate, direct

role for the pteridine. It is unlikely that non-specific hydrogen donors would display any binding with the enzyme. It would also be of value to investigate eye color mutants for abilities to support the conversion of tyrosine to dopa. It was found in the cases where various eye color mutants were screened for phenylalanine hydroxylase activity that they possess wild type levels of activity, presumably utilizing an active pteridine in the process. Since the pathway leading up to eye pigmentation is multi-stepped, it is probable that the active pteridine is synthesized and the block causing the particular phenotype occurs after these critical synthetic steps. $DMPH_4$ does not absorb light in the visible region so that only mutants possessing pigment-free eyes would be of value. If the pteridine serves such a necessary function, as it seems to do at least in the phenylalanine hydroxylase system, a mutation which results in a complete absence of pteridine would likely be lethal. Therefore,use must be made of conditional mutants.

It is obvious that tyrosinase, in the presence of an appropriate reductant system, is capable of meeting the exigencies of the sclerotization pathway. Dopa can be protected from further irreversible oxidations that lead to melanization, and, consequently, would be allowed to participate in sclerotization, most likely by decarboxylation

and acetylation to yield N-acetyl-dopamine. It should be kept in mind that reductants such as ascorbate and DPNH may be serving in ways other than merely maintaing dopa in the reduced state, as has been suggested by Krueger (75,76).His data indicate that the modes of action of these two factors differ from the manner in which catalytic amounts of dopa stimulate the monophenolase reaction. It appears that they do not merely favor dopa accumulation because, as Krueger pointed out, this regimen would require a short lag before any dopa accumulated; no lag was observed in these experiments. Additionally, the rate of the reaction in the presence of either factor is greater than when dopa is used in their stead. There is also an additional consumption of oxygen when either ascorbate or DPNH is used that cannot be accounted for in the products of the reaction. Utilizing mouse tyrosinase, Pomerantz presented data somewhat at odds with these. He found that ascorbate, at a final concentration of 12 mM, could not entirely obviate the lag, whereas $DMPH_4$ at 2.3 mM completely abolished it, as did dopa at 0.04 mM. (79). Pomerantz also suggested, without any data to substantiate the claim, that $DMPH_4$ may serve to initiate the reaction and dopa serves to keep the process going. No mention was made if the mouse tissue contained a pteridine. Nevertheless, this is an attractive hypothesis because it allows for

potential modulation of activity by a rather specific compound, tetrahydropteridine. Of course, either $DMPH_4$ or another reductant, such as, ascorbate or a reduced pyridine nucleotide, must be present to protect dopa from subsequent oxidation by the dopa oxidase activity, thereby allowing it to participate in sclerotization. It is obvious that any compound with the appropriate reduction potential is capable of stimulating the hydroxylation reaction, but if tyrosinase were the enzyme responsible for providing dopa for sclerotization, one would expect that a more specific regulatory system would be operative for this critical reaction.

The nature of peak I material

Although there are no direct data bearing on the identity, much less significance, of the material in Peak I, a number of experiments did yield useful information.

The most noteworthy features of peak I material are that it is derivable from dopa and the reaction is stimulated by $DMPH_4$. Most of the information accrued came from experiments using tyrosine as the substrate so that the ultimate substrate (dopa) for the reaction was a limiting factor.

The levels of peak I material and dopa respond to $DMPH_4$ in a different manner. Reactions carried out either in the presence of a high concentration of pteridine (8 mM) or in its absence result in a lack of accrual of peak I material. The

optimal concentration of pteridine falls somewhere in between, the ratio of factor to enzyme being critical. The data displayed in Table 4 show that peak I material responds to $DMPH_4$ in much the same way as does the origin material. However, in most cases, the synthesis of origin material was maximal at the lowest concentration of the pteridine, whereas a slightly higher concentration of the factor was optimal for synthesis of peak I material.

It is of considerable interest that peak I material arises non-enzymatically in a $DMPH_4$ dependent reaction. In the situation depicted in Table 4, 8 mM pteridine inhibits this synthesis, whereas 0.8 mM is optimal. Quantitative results with the non-enzymatic reactions were poorly reproduced, part of the problem residing in the fact that chromatography was often poor in this region and the level of activity low,so that definition of peaks was difficult. It will also be noted in Figure 14 that commercial tritiated dopa contains peak I as a contaminant, which implies that it is readily derivable from dopa.

DTT inhibits the synthesis of peak I material. As noted in Table 4, the inhibition was complete, whereas the syntheses of both dopa and origin material were not as severely inhibited. This suggests that the enzymatic activity responsible for the synthesis of peak I material is distinct from the other two.

In a number of experiments, peak I material was accumulated in the presence of DTT, and, in fact, in the original experiments on this system, DTT was present at a concentration of 1 mM and peak I material was consistently observed. The rate of synthesis of peak I material is severely inhibited in the presence of DTT in much the same way as is dopa.

The enzyme responsible for the synthesis of peak I material behaves similarly to the enzymes involved in origin material and dopa syntheses upon ammonium sulfate fractionation. It is of interest to note in the case where the 0-40% fraction was used, $DMPH_4$ inhibited the synthesis of peak I material. When the two fractions were combined, pteridine enhanced the synthesis in the usual manner. The reason for this discrepancy is not known.

Banded tyrosinase supports the synthesis of peak I material in a $DMPH_4$ stimulated reaction. Hence, the enzymatic activity responsible for the synthesis of peak I material is associated with the tyrosinase complex.

Ascorbic acid used in the same concentration range as $DMPH_4$ is inhibitory to the synthesis of peak I material. Very small quantities do allow for some synthesis, but no enhancement over the non-addition case was observed. When both fac-

tors were present, ascorbic acid antagonized the stimulatory effect of the pteridine. These results indicate that $DMPH_4$ is not exerting its enhancement effect on the synthesis of peak I material through its reductant properties, because ascorbate, at a wide range of concentrations, severely inhibits the reaction. $DMPH_4$ used at concentrations an order of magnitude higher results in total inhibition. At these concentrations, the reductive effect of the pteridine overrides its stimulatory one. In the case where mushroom tyrosinase was used as the source of enzyme, a peak of activity arose in the area where Drosophila peak I material chromatographs when present. Unfortunately none appeared in the experiment described in Figure 20, so an unequivocal statement concerning the identity of the material cannot be made. Assuming that the material does correspond to peak I material, it is obvious that either $DMPH_4$ or ascorbate is necessary for the accumulation of the compound. Similar experiments should be carried out using various concentrations of the factors to determine their relative effects on the reaction.

These data suggest that the material in peak I may be trihydroxylated phenylalanine. The existence of such a compound in insects has not been reported. The facts that the accrual of peak I material is dependent upon $DMPH_4$ and that the reaction occurs non-enzymatically in the presence of the

pteridine substantiate this claim. Furthermore, banded tyrosinase is capable of catalyzing the reaction. The other position ortho to the original para hydroxyl group should be susceptible to a hydroxylation reaction. It is not obvious why a reducing agent should antagonize this reaction since an electron donor is required for the reaction in the same way as the hydroxylation of monophenols. If, in fact, this trihydroxylated compound does exist, the physiological significance of it is not clear. This material may serve as a substrate for dopa oxidase since its level of accumulation falls off with a decrease in $DMPH_4$ concentration, a condition which favors dopa oxidase activity. The material in peak I is not an obligatory intermediate in the dopa to melanin reaction because the decrease in its level is not large enough to account for the increase in the level of origin material between the two concentrations of pteridine. Furthermore, it is actively accumulated in assays where dopa oxidase is active.

The accrual of peak I material is not affected by the presence of cycloheximide. Drosophila larvae are capable of synthesizing cycloheximide resistant peptides, so the possibility that peak I represents peptides is still open (86). However, it is not obvious why $DMPH_4$ would stimulate this kind of reaction.

Peak I material could correspond to the ketocatechols

(or precursors) that are isolated from Drosophila pupal cases (100). These compounds appear to be actively involved in the sclerotization process. No further work was performed to evaluate this notion.

Dopamine and N-acetyl-dopamine, which are likely products of further dopa metabolism, do not chromatograph in this region.

Peak I merely could represent a gratuitous product of the in vitro system. Obviously, it is of importance to search for this compound in vivo . Occasionally, when C^{14}-phenylalanine was injected into larvae, a small peak of activity was observed in this region of the chromatogram. Injection of tyrosine and dopa would be more meaningful.

Relationship of "tyrosine hydroxylase" with other aromatic amino acid hydroxylases

Banded tyrosinase, which is capable of catalyzing the $DMPH_4$ dependent synthesis of dopa, does not possess phenylalanine hdyroxylase activity. Of course, since Drosophila phenylalanine hydroxylase is an extremely unstable molecule, these negative results are not conclusive. One might postulate that these two enzymes would be closely associated since the product of one serves as substrate for the other. However, there is good evidence that suggests tyrosine is stored as either tyrosine-O-phosphate or in the form of a tyrosine rich protein so that these economic considerations are not compelling.

There are also some data which suggest that tyrosine (and phenylalanine) hydroxylase possesses tryptophan hydroxylase activity. Crude Drosophila extracts were assayed for their abilities to convert tryptophan to 5-hydroxytryptophan and none was found.

In summary, it appears that the tyrosinase complex provides dopa for sclerotization since the only "tyrosine hydroxylase" activity observed in vitro is associated with tyrosinase. The facts that this activity co-purifies with banded tyrosinase and, also, behaves similarly to this complex upon ammonium sulfate fractionation substantiate this notion. Tyrosinase displays heterogeneity with respect to substrate utilization, one species being relatively ineffecient in catalyzing the overall tyrosine to melanin reaction, a process which is conducive for dopa accumulation. That two species of tyrosinase appear to exist in extracts (in the case with ebony) could account for the observation derived from kinetic experiments that there are at least two pathways capable of synthesizing dopa from tyrosine. The tyrosinase responsible for providing free dopa for sclerotization still requires a reductant and $DMPH_4$ is a likely candidate for this function.

The possibility that there is a distinct tyrosine hydroxy-

lase cannot be ruled out. If this enzyme does exist, it would likely be similar to phenylalanine hydroxylase in analogy to the mammalian system. Drosophila phenylalanine hydroxylase is extremely unstable, so, if tyrosine hydroxylase shares this property with the enzyme, it would likely go undetected in many of the experiments reported here where prolonged purification procedures were employed.

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