THE ROLE OF TYROSINE IN THE TANNING AND SCLEROTIZATION OF THE PUPARIUM OF *DROSOPHILA MELANOGASTER*

Thesis by

William Jack Driskell

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1974

(Submitted September 20, 1973)

ACKNOWLEDGEMENTS

I wish to thank Dr. Mitchell for his patience, good humor, and guidance. His fine human qualities and excellent scientific example have helped me immeasurably during years in which I have not always had a clear direction.

Others whom I would like to thank are:

John Smart, for the SDS gels and much help and advice.

Alan Blumenthal, for his friendship and kindness in showing me techniques.

Jack Geltosky, a good friend and fellow traveler.

The Public Health Service for support.

And all the many other good people I have known at Caltech.

ABSTRACT

The cuticle of insects is sclerotized and tanned by crosslinks between proteins formed by tyrosine derivatives. The pathway from tyrosine to the catechols incorporated into the crosslinks in the puparium of Drosophila, the structure of the crosslink, and other aspects of sclerotization and tanning of the Drosophila puparium were studied.

At puparium formation there is an influx of tyrosine into the puparium, where it is incorporated as a catechol. The turnover of this catechol is very rapid. Synthesis of N-acetyldopamine, the sclerotizing catechol of Calliphora, was observed. No storage form in the larva for the tyrosine incorporated at puparium formation exists other than the pool of free tyrosine and tyrosine-O-phosphate. No differences in the larval and prepuapl tyrosine metabolisms of wild type and ebony were observed.

Puparia were degraded by acid treatment and by enzymolysis and the degradation products were analyzed to determine the nature of the sclerotizing crosslink. A ketocatechol was extracted in considerable quantity from the puparium by acid. The catechol extractable as a ketocatechol is incorporated into the puparium at puparium formation. The amount of this catechol incorporated is equal to the amount of free tyrosine and tyrosine-O-phosphate lost from the hemolymph at puparium

formation. The amount and type of ketocatechol as well as other tyrosine derivatives extracted by acid from the puparium are the same for ebony and wild type.

Enzymolysis of the puparium yielded soluble products which contain the crosslink extractable as ketocatechol. These products were fractionated and found to be heterogeneous.

Possible structures and syntheses of the sclerotizing crosslink are discussed.

TABLE OF CONTENTS

INTRODUCTION

Exoskeleton

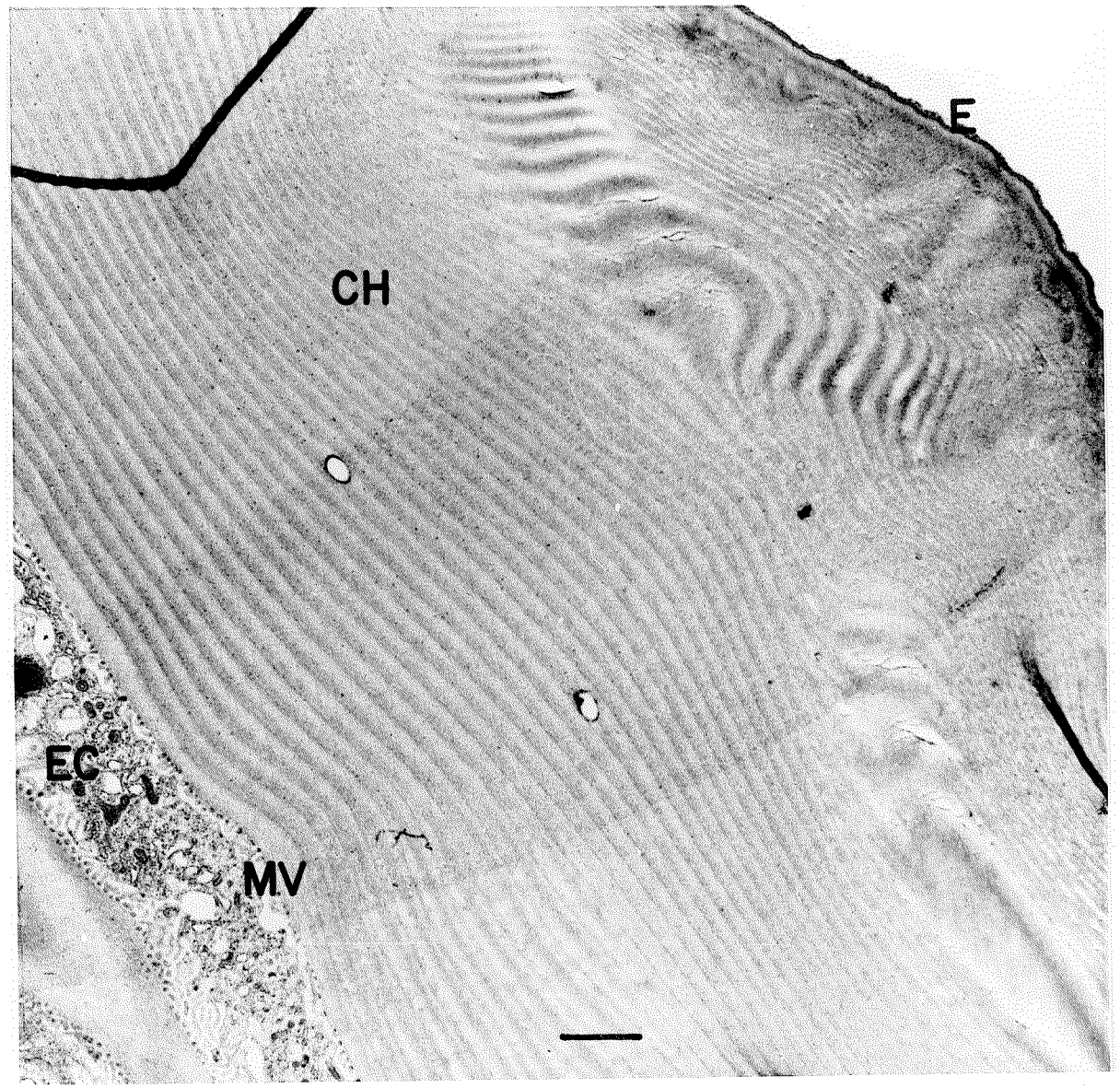
The exoskeleton of arthropods is a more or less inert but multilayered structure which is secreted by a single layer of underlying epithelial cells over the exterior including invaginations, such as, the buccal cavity and foregut, the trachea, the genital ducts, and various glands that open upon the surface (1).

The epidermis is seen at its full development only when new cuticle is being laid down; at other times it is very attenuated. The epidermis (see Figure A) is composed of epidermal cells, dermal cells, trichogen cells, oenocytes, and a basement membrane (1, 2).

The epicuticle, only a few microns thick, is the outer layer of the cuticle. The epicuticle contains an inner and outer epicuticle. Outside the outer layer of the epicuticle is deposited a wax layer above which is a cement layer. In some cases, the cement layer is diffuse and permeated by the wax layer (3). The epicuticle is an important factor in water balance in insects.

The layer below the epicuticle is the procuticle, which is divided into two sublayers: the upper, sclerotized exocuticle and the lower, flexible endocuticle. The rigidity of the

Figure A. Drosophila larval cuticle, very late third instar 119 hours from egg laying. Low magnification showing an entire cross section of integument just prior to puparium formation. Marker, 2 microns; EC, epithelial cells; MV, microvilli; E, epicuticle; CH, chitin. This micrograph courtesy of H.K. Mitchell .


E
CH
EC
MV

exocuticle makes the cuticle an efficient skeleton, the firmness of which is not dependent on the turgor pressure of internal water as is the case with primitive insects. Unlike the epicuticle, which appears to be uniform over the whole body, the procuticle has many different manifestations. It can be very thin and delicate as in the terminal branches of the tracheal system and in the wing veins, and it can be thick and horny with a comparatively thick exocuticle as with certain beetles. At joints between segments the procuticle may be considerably thinner than adjacent procuticle; or the exocuticle may be absent; or there may be indentations of endocuticle into the exocuticle (1, 3). At the joints, the cuticle also appears to be more hydrated than elsewhere (6).

Arthropods, also, have an elastic procuticle composed of chitin and the elastic protein resilin. The resilin procuticle is used for the storage and release of mechanical energy. The resilin of the thorax and wing hinges decreases the energy expended in flight. The flea's jump is powered by the release of a resilin spring (5, 7).

Ultrastructure of the Cuticle

The ultrastructure of the cuticle is not well understood. The chitin component is most amenable to ultrastructural studies because of the visible pattern of chitin fibers.

The chitin of the procuticle has an obvious lamellate pattern. In many insects lamellate cuticle is laid down during the day and non-lamellate cuticle is laid down at night (5). Neville and Luke (8) distinguish between a helicoidal pattern of chitin fibers which is responsible for the lamellate appearance of the cuticle and a non-lamellate, unidirectional arrangement, in which the fibers in the next layer all point in a different predetermined direction. They suggest that the helicoidal pattern represents spontaneous self-assembly, the natural crystallized form of chitin; whereas, the unidirectional arrangement requires the closer control by epidermal cells.

The spatial relationship of protein to chitin is not clear. It has been suggested that the chitin fibers are embedded in a matrix of protein as the glass fibers of fiberglass are embedded in resin (5).

The lipids of the outer epicuticle appear to be oriented so that the lipid chains are perpendicular to the cuticle surface (4).

Composition of the Cuticle

The wax layer of the epicuticle varies in character from a fairly soft structure to a hard crystalline structure. The softer waxes appear to have a higher content of hydrocarbons

than the harder ones (1). The lipid extractable material from the wax layer of the Mormon cricket contains 48-58 percent free fatty acids; 2-3 percent cholesterol; and 12-14 percent of unidentified organic compounds (9). The wax layer of the cockroach contains mostly unsaturated hydrocarbon. The hard wax layer of Tenebrio is made up of 10 percent hydrocarbons and 55 percent long chain diols (10). The powdery wax layer of the silkworm Samia is composed of two straight chain, unsaturated alcohols (11).

Both epicuticle sublayers lack chitin. The outer epicuticle is the most chemically resistant layer of the cuticle. It appears to be a highly stabilized protein which contains lipid and phenolic substances (4, 13).

The procuticle is composed mainly of protein, chitin, and lipid. The proportion of the three components varies among different procuticles; on the average, the amount of protein is slightly more than the amount of chitin, especially in the exocuticle. The exocuticle also appears to contain more lipid than the procuticle.

The proteins of the procuticle are fairly insoluble in aqueous media even before sclerotization. Various studies with the proteins that are soluble show that the procuticle contains many different kinds. Fourteen soluble proteins are present in the cuticle of the cockroach at one stage of ecolysis (14). The many different kinds of proteins prob-

ably account to a large extent for the variability in cuticle: none of the proteins extracted from the solid cuticle of the locust is the same as any extracted from intersegmental cuticle (4). Resilin is probably the best known cuticle protein, but its study is hampered because it is present only in the crosslinked insoluble state (7).

Chitin is used as a structural material among most invertebrates, some amoebae, and some diatoms (4). Chitin is made up of long, linear chains of aceylated glucosamine residues linked with 1,4-beta-glucosidic linkages. The crystallographic form, alpha, is insoluble in everything except strong mineral acids which hydrolyze it. The beta form of chitin is soluble in various substances including formic acid. The alpha form is used in the cuticle as well as in all permanent or non-growing shell structures (1, 15).

Cuticle Formation

As the insect grows, the cuticle must be shed and replaced by a larger one from time to time. The new cuticle is extruded outside first from the epidermal cells. The first structure formed is the outer layer of the epicuticle; Locke (16) calls this layer the most important layer of the cuticle because its shape appears to determine the shape of the finished cuticle. How the delicate shaping of this layer is controlled by the epidermal cells is an open question. Soon

after the outer layer is formed, the inner layer of the epicuticle is laid down. The wax layer and the cement layer are deposited soon after molting.

While the epicuticle is being completed, the procuticle is being laid down. How it is done probably varies among different species, but a mechanism generally accepted is that proposed by Leydig, which states that the cuticular substance is secreted around the filiform outgrowths of the epidermal cells, which later become pore canals (1). There is evidence of fibrillar chitin material inside the epidermal cells indicating that the chitin synthetase is secreted along with the substrate or is localized on the outside of the plasma membrane of the epidermal cells (6, 16). The procuticle continues to become thicker long after the molt. The procuticle of Drosophila thickens throughout the third instar until the whole procuticle is tanned and hardened to form the puparium (17).

The hardening and tanning of the exocuticle is a secondary process that takes place after molting. The enzymes necessary for hardening and tanning are evidently incorporated into the cuticle later. Stevenson and Adamako (18) observed the location of phenoloxidase activity in frozen sections of crayfish cuticle by incubating the sections in phenol substrates. Presence of the activity in newly formed cuticle indicates that the phenoloxidase is built into the cuticle.

Studies by Mitchell *et al.* on *Drosophila*, using autoradiography and electron microscopy, provide evidence that there is no movement of large molecules, such as proteins and polysaccharides, through the cuticle layer (17).

Little is known about the extent to which enzymes and structural proteins of the cuticle are synthesized in the epidermal cells. Large vesicles commonly found in the epidermal cells might be the means for transporting protein synthesized in other parts of the animal (4). Some proteins of the cuticle appear to be identical to proteins in the hemolymph (14). Phenoloxidase in an inactive form is present in the hemolymph, but it is not certain that this one is the same as the one incorporated into the cuticle. Resilin does appear to be synthesized in the epithelial cells (19).

The type of cuticle formed may depend partially on the substrates made available by the epidermal cells to the enzymes of the cuticle. In studies with *Drosophila* intersexes, Stern (20) found that the pigmentation of the cuticle depends on the genetic type of the underlying epithelial cells. This finding indicates that the pigmentation is controlled by the quantity and quality of the phenoloxidase substrate secreted by the epidermal cells, if it is assumed that the phenoloxidase is built into the cuticle.

The driving force for movement of substrate through the cuticle is not known. In *Drosophila* it is suggested that the

muscular contraction and high internal pressure that occurs at puparium formation is responsible for forcing substrate into the cuticle (17). The only accurate measurement of internal pressure in insects was done by Cottrell (43), who measured the internal pressure of Calliphora during imaginal ecdysis and found a maximum pressure of 96mm Hg during wing expansion. Fristrom (69), working with Drosophila, found that the drop in external pressure necessary to produce premature head emergence was about 450mm Hg.

Theories on the Sclerotization and Tanning of Insect Cuticle

The structure of the covalent crosslink that stabilizes hard or sclerotized cuticle, which composes the exocuticle and, in the case of higher Diptera, the puparium, is still unknown. The structure of stabilizing covalent crosslinks in some other extracellular structures is known. Most covalent crosslinks among proteins occur extracellularly. The disulfide crosslink occurs both extracellularly and intracellularly. The following are some examples of covalent crosslinking of protein:

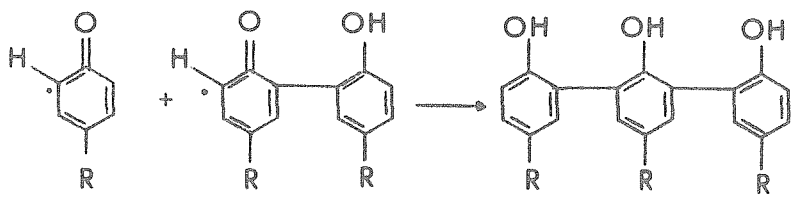
Keratin This group of proteins is present in hair, nail, feather, and skin of vertebrates. Keratin is crosslinked by the disulphide bond (7).

Collagen The protein of connective tissues, collagen, is the most abundant protein of higher animals, occurring in skin, tendons, blood vessels, bone, teeth, cornea, and vitreous humor. Collagen crosslinks are the result of reactions between aldehyde derivatives of lysine residues and other amino acids in adjacent collagen molecules. The different crosslinks formed can bond together two, three, or four collagen chains (21, 62).

Elastin The unusual amino acids desmosine and iodo-desmosine serve as crosslinks in elastin, a protein of the elastic fibers of vertebrates. Both contain a pyridinium nucleus with four aliphatic side chains, each with a terminal carboxyl group. Possibly, each of these amino acids can crosslink as many as four peptide chains (7, 22).

Bacterial cell wall Diaminopimelic acid, which crosslinks lysine residues, is the only covalent crosslink identified (7, 23).

Reselin In insects, the only cuticle protein crosslink definitely established is the di- and trityrosine crosslink in reselin. Concentrated acid digests of reselin yield dityrosine and trityrosine. Andersen postulates that the link is formed when tyrosine residues in adjacent peptide chains are oxidized to a free radical state and react (7):



Noncovalent crosslinks are also important in stabilizing insect cuticle. With crustaceans and a few species of insects (1), the hardening of parts of the cuticle is accomplished by the incorporation of lime producing electrostatic crosslinks. Aspartic acid of cuticle protein seems to be involved (25). Lime hardened cuticle is probably not suitable for insects because it is considerably more rigid than normal insect cuticle.

The stability of all arthropod cuticles is partly due to the presence of hydrogen bonds. During sclerotization the cuticle shrinks, increasing hydrogen bond formation by decreasing the hydrated domain of the macromolecules (26). The hydrogen bonds account to some extent for the high resistance of insect cuticle to enzyme degradation (27).

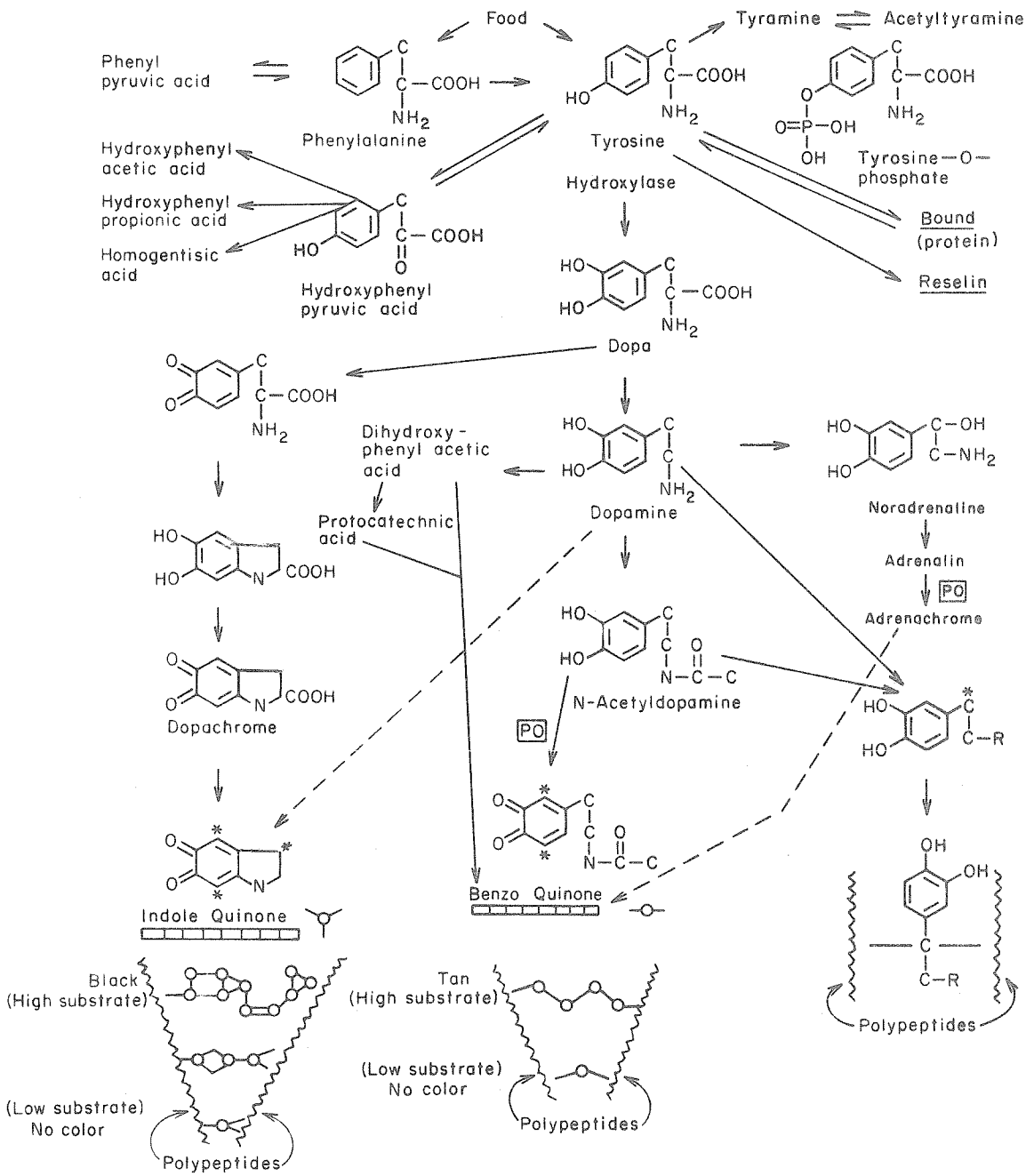
The generally accepted explanation for cuticle hardening, which derives from Pryor's work (28, 29) on the ootheca of the cockroach, has been the quinone crosslinking of cuticle proteins. The ootheca is formed from the interaction of two secretory glands: one gland secretes a solution of protein and phenoloxidase; the other gland was believed to secrete

a solution of protocatechuic acid. The protocatechuic acid is supposedly oxidized to a quinone when the two solutions are mixed; the quinone than crosslinks protein, hardening and tanning the ootheca. Later, Brunet and Kent (30) found that the protocatechuic acid and the phenoloxidase are secreted from the same gland; however, the protocatechuic acid is in the form of a glucoside. A glucosidase in the other gland frees the protocatechuic acid when the two solutions are mixed.

According to the quinone theory, orthoquinones, produced by cuticular phenoloxidases, polymerize and form covalent bonds with cuticle protein and with each other, both hardening and tanning the cuticle. Orthoquinones can react with each other and with free amino, imino, and sulfhydryl groups of the amino acid residues of protein by means of two electrophilic active sites on the quinone ring (31). The substrates for the phenoloxidase, according to the earlier versions of the theory, are acidic, deamination products of tyrosine, such as, protocatechuic acid and homogentisic acid (33)(see Figure B).

An offshoot of the quinone theory is the theory of "autotanning", which states that the tyrosine residues of cuticular protein are oxidized <u>in situ</u> to quinones, which react with free amino or other reactive groups in nearby peptide chains (32). The length of the crosslink is restrict-

Figure B. A scheme of tyrosine metabolism in insects incorporating current theories about the sclerotizing crosslink and showing how sclerotization and melanization may be related.


Food
Tyramine
Acetyltyramine
Phenyl pyruvic acid
Phenylalanine
Tyrosine
Tyrosine—O—phosphate
Hydroxyphenyl acetic acid
Hydroxyphenyl propionic acid
Homogentisic acid
Hydroxyphenyl pyruvic acid
Hydroxylase
Bound (protein)
Reselin
Dopa
Dihydroxy-phenyl acetic acid
Protocatechnic acid
Dopamine
Noradrenaline
Adrenalin
PO
Adrenachrome
Dopachrome
N-Acetyldopamine
Indole Quinone
Benzo Quinone
Black (High substrate)
(Low substrate) No color
Polypeptides
Tan (High substrate)
(Low substrate) No color
Polypeptides
Polypeptides

ed in this theory because of the inability of the tyrosine residues which become quinones to polymerize.

Karlson and his colleagues (34, 35, 36) proposed that a substrate for the cuticular phenoloxidase is the decarboxylation product of tyrosine, N-acetyldopamine. N-acetyldopamine is considered to be incorporated into the cuticle as a whole molecule. Karlson pointed out that the acetyl group of N-acetyldopamine precludes the formation of indolequinones, which have three active sites for crosslinking and are capable of producing more darkening than benzoquinones because of more extensive polymerization. The phenoloxidase substrate in lighter cuticle might be mostly N-acetyldopamine and that in darker cuticle a compound that can form an indole quinone such as dopamine (see Figure B).

Andersen proposed a crosslink for sclerotized cuticle different from the quinone crosslink but involving tyrosine derivatives. From cuticle hydrolyzed with boiling 1N hydrochloric acid, considerable amounts of a substance identified as a ketocatechol are released (37). The structure of the ketocatechol molecule led Andersen to propose a crosslink involving oxidation of the beta-carbon in the aliphatic side chain of dopamine or some metabolite of dopamine (38, 39).

Lipke proposed a scleroterizing crosslink that does not involve tyrosine derivatives. In this theory chitin and protein are covalently crosslinked in an unspecified

way by neutral sugars (40, 41).

Evidence for the Theories of Sclerotization and Tanning

There is considerable evidence that tyrosine or metabolites of tyrosine are involved in sclerotization and tanning. In Drosophila, during growth no general pattern in the variation of individual amino acids can be recognized with the exception of tyrosine and proline. These show continuous increase, especially toward puparium formation,when tha values are expressed per unit body weight. The same pattern is true of mosquito larvae, the housefly, and other insects (42).

Tyrosine and tyrosine derivatives do appear to show up in the cuticle during hardening and tanning. Dennel (44), using staining techniques, found that there is an influx of polyphenol staining material into the cuticle during puparium formation of the blowfly. When labeled tyrosine is injected into the larvae of Calliphora 80 percent of the total activity can be recovered from the puparium (45). Using autoradiographic techniques with Drosophila, Mitchell et. al.(17) showed that when tritiated tyrosine is injected into the larvae 20 hours before puparium formation the label accumulates in the epithelial cells just below the cuticle. Immediately before puparium formation considerable mobilization of the label appears at the cell-cuticle interface. During the first 3

hours of puparium formation the labeling becomes more dense in the cuticle with the density of the label higher toward the outside.

In many insects studies to determine the nature of the substrate of cuticular hardening and tanning enzymes have been made. Early investigators were not very successful in finding suitable free dihydroxyphenols. In some species no dihydroxyphenols could be found (33). In other species, the dihydroxyphenols present such as homogentisic acid and protocatechuic acid are not very easily oxidized by phenoloxidases. Dihydroxyphenols in a masked form as glucosides as in the secretion of the ootheca synthesizing gland of the cockroach were considered, but none was found.

It now appears that two processes act on tyrosine in insects. One is the deamination of tyrosine to acidic products, which were the ones most often found by early investigators. The deamination process goes on throughout the life of the insect. The second process is the decarboxylation of tyrosine and its derivatives and is connected with molting and puparium formation; the decarboxylation is controlled by the hormone ecdysone as shown by Karlson and Sekeris (34, 46). The nature of a tyrosine metabolite that is incorporated into the cuticle has been established by Karlson and his colleagues (34, 35, 36) working with

Calliphora. N-acetyldopamine is accumulated and disappears abruptly at puparium formation. Regardless of which carbon atom is labeled, the same proportion of label is incorporated into the cuticle, indicating that N-acetyldopamine is incorporated as a whole molecule. The compound is one of the best substrates for Calliphora phenoloxidase. N-acetyldopamine has been found in other insects including meal worms, crickets (36), cockroaches (47) and Drosophila (17).

The question of the source of tyrosine used in cuticle hardening arises. In Drosophila larvae, there is a pool of free tyrosine and tyrosine-O-phosphate. The decarboxylation products of tyrosine are present in almost undetectable quantities. Tyrosine-O-phosphate, identified by Mitchell (48), is the most prominent free tyrosine derivative; the compound is present throughout the life of Drosophila, approaching the concentration of tyrosine just before puparium formation. Both the levels of tyrosine and tyrosine-O-phosphate fall sharply immediately after puparium formation (49). The role of tyrosine-O-phosphate is not known. It is not metabolized at the phosphate level at puparium formation but follows the same route as tyrosine (50). Tyrosine-O-phosphate has also been found in the blowfly (51). Fraenkel et al.(51) concluded from labeling experiments that the pool of free tyrosine and tyrosine-O-phosphate in the blowfly is not sufficient to

account for all the tyrosine used in sclerotization. A protein precursor, which is hydrolyzed at the time of sclerotization to furnish tyrosine for the cuticle, was suggested. Another possible storage form is a tyrosine rich protein that has been found in large amounts in blowfly adults (31). Phenylalanine as a reservoir for tyrosine is probably not important. Drosophila does have a phenylalanine hydroxylase that has peak activity at puparium formation (52); however, the pool size of phenylalanine is small compared to that of tyrosine and remains constant through puparium formation (42).

The evident involvement of tyrosine in sclerotization and tanning is necessary but not sufficient evidence in support of the quinone theory of crosslinking. The best evidence, the isolation of the crosslink, is lacking. One reason that the quinone theory has been widely accepted is that quinones obviously can harden and tan protein: gelatin placed in a quinone solution will become tan and hard.

To oxidize diphenols to quinones, phenoloxidases in the cuticle at the time of hardening and tanning are necessary. Stevenson and Adomako (18) observed the location of phenoloxidase activity in frozen sections of crayfish cuticle by incubating the sections in phenol substrates. Yamazaki (53) found phenoloxidase activity in the cuticle of the white prepupa of Drosophila. In Drosophila cuticular phenol-

oxidase activity can also be demonstrated by the blackening of the cut end of a tracheal tube when immersed in a dopa solution (54). Unsclerotized cockroach cuticle contains a soluble phenoloxidase (55).

The dark cuticular pigment of insects is almost certainly a product of phenoloxidase. The dark pigment has always been called melanin, a polymer of indole quinones, because insect cuticle often darkens when placed in a solution of phenols. The melanic nature of an insect pigment was first demonstrated by Hackman (56) in 1967. Analysis of the degradation products from the pigment of Lucilliana Cuprina shows the presence of pyrrole di-, tri-, and tetracarboxylic acids, permitting the pigment to be classified as a melanin. Fogal and Fraenkel found that both the cuticle and puparium pigments of the blowfly are melanins (57, 58). There is no evidence to say that melanization and sclerotization are the same process.

A puzzling aspect of pigmentation is the involvement of the compound beta-alanine. Jacobs (59) found considerable amounts of beta-alanine in Drosophila pupal cases. The Drosophila mutant ebony, which has a white rather than a tan pupal case, does not incorporate beta-alanine (60). Hodgetts (61) showed that ebony synthesizes beta-alanine but is unable to incorporate it into the pupal case; black, which also has a white pupal case, does not accumulate beta-alanine.

Evidence for a covalent cuticle crosslink involving tyrosine but different from a quinone type crosslink comes from Andersen's laboratory (37, 38, 39). Ketocatechols that are released from the cuticle by boiling 1N hydrochloric acid would not be expected to be released from a quinone crosslinked cuticle; quinone crosslinks are not hydrolysed by even concentrated hydrochloric acid. Cuticular proteins become increasingly insoluble as the amount of the substance that can be extracted as ketocatechol increases during sclerotization, suggesting that the substance extracted as a ketocatechol is involved in sclerotization. Andersen (38) postulated that the formation of the crosslink involves oxidation of the beta-carbon atom in the aliphatic side-chain of dopamine or some metabolite of dopamine. Keto-catechols can be hydrolyzed from the cuticles of several insects, but not from the cuticles of crustaceans.

According to the ideas of Lipke (40, 41), the cross-linking of chitin, as well as protein is involved in sclero-tization. Evidence for the crosslinking of chitin to chitin or chitin to protein by means of neutral sugars comes from experiments involving degradation of the cuticle. Mannose, galactose, and glucose were identified in acid hydrolysates; the titre of the bound hexoses increased as sclerotization proceeded. Partial acid hydrolysis afforded such fragments as mannosylated chotodextrins and seronyl- and threonyl-

glycopeptides.

Research Objectives

The object of the experimental work of this thesis was to investigate the metabolism of tyrosine and the involvement of tyrosine and its derivatives in the sclerotization and tanning of the pupal case of Drosophila melanogaster. The investigation was conducted mainly by means of two experimental approaches: tracing the fate of radioactively labelled tyrosine and tyrosine derivatives in larvae, prepupae, and pupae; and degrading the pupal case with acids and enzymes and isolating tyrosine derivatives.

It was hoped to learn something about the following problems: (1) the role of tyrosine-O-phosphate; (2) the identity of tyrosine derivatives incorporated into the cuticle at puparium formation; (3) whether there exists a storage form for the tyrosine used at puparium formation other than free tyrosine; (4) the type of crosslinks that are formed from tyrosine derivatives in the cuticle when the sclerotization and tanning of puparium formation occurs.

Drosophila was especially useful for these experiments. The existence of cuticle color mutants such as ebony and black allows interesting comparisons of tyrosine metabolisms. The tanning and sclerotization of the larval cuticle to form the pupal case provides a well defined system for comparisons

between sclerotized and tanned cuticle and unsclerotized and untanned cuticle.

MATERIALS AND METHODS

Stocks and Culture Methods

Drosophila melanogaster of the *ebony*, *black*, and Oregon R wild type were raised as described by Mitchell and Mitchell (63).

Chemicals and Enzymes

Uniformly labeled ^{14}C-L-Tyrosine (380mCi/mmole), uniformly labelled ^{14}C-L-Leucine (270mCi/mmole), Dihydroxyphenylethylamine-1-^{14}C. HBR (50mCi/mmole), Beta-Alanine-1-^{14}C (50mCi/mmole), and Dihydroxyphenylethylamine-2-^{3}H(5Ci/mmole) were obtained from New England Nuclear. 3,4-Dihydroxyphenylalanine-2,3-^{3}H (1Ci/mmole) was obtained from Amersham-Searle. Pronase, Type VI from *Streptomyces griseus*, was obtained from Sigma; and chitinase was obtained from Calbiochem (Lot #73217).

Injection

Materials were injected into larvae, prepupae, and pupae by the method worked out by Mitchell and described by Boyd (64).

Synthesis of Tyrosine Derivatives

N-acetyldopamine N-acetyldopamine was synthesized after

a method suggested by H. K. Mitchell (65). 400 Mg of dopamine. HCl were combined in a test tube with 4 ml of pyridine (dry) and acetic anhydride (0.3 ml) was added. The mixture was allowed to stand at room temperature for 6 hours and then evaporated over phosphorus pentoxide. The sample in water was applied to a 1 x 20 cm Dowex 50 (H^+ form) Column. Through the column was run 40 ml of 0.1N HCl; the sample was eluted with 0.2M ammonium formate in 4M formic acid. The solution containing the sample was evaporated over phosphorus pentoxide. The sample was further purified over a P-2 column; elution was effected with distilled water.

The same procedure was followed for the synthesis of N-acetyldopamine-1-^{14}C and N-acetyldopamine-2-^{3}H except that localization of the sample was by radioactive counting rather than by U.V. absorption. The initial mixture consisted of 100 microliters of pyridine, 8 microliters of acetic anhydride, and 10 microcuries dopamine-1-^{14}C (50mCi/mmole) or 100 microcuries dopamine-2-^{3}H (5mCi/mmole), which had been dried in the reaction vial. The excess of acetic anhydride did not adversely affect the synthesis.

Tyrosine-O-phosphate Uniformly labelled ^{14}C tyrosine-O-phosphate was synthesized according to the method of Lunan and Mitchell (49) with certain modifications because of the very small amounts of tyrosine used in order to yield high

specific activity. Because of the difficulty of handling phosphorus pentoxide with its property of avidly absorbing water from the air, a small unweighed quantity of phosphorus pentoxide was added to the reaction vial containing 50 microliters of pyridine and uniformly labelled ^{14}C tyrosine in various quantities; the reaction vial was quickly closed. Phosphorus pentoxide added was always in considerable excess of the very small amount of tyrosine. The reaction mixture was incubated usually for 15 minutes. An impurity, apparently tyrosine-O-diphosphate, which was usually formed in a quantity about equal to that of tyrosine-O-phosphate, was easily separated from tyrosine-O-phosphate on a Dowex 50 column (H^+ form) eluted with distilled water. The specific activity of the tyrosine-O-phosphate was as high as 380mCi/mmole. U.V. spectra (when possible), paper chromatography and high voltage paper electrophoresis were used to verify the purity of radioactive tyrosine-O-phosphate.

Ketocatechol The ketocatechol 2-hydroxy-3', 4' dihydroxyacetophenone was synthesized by the method of Fodor and Kovacs (66).

Storage

Larvae, prepupae, and pupae of various ages were quickly frozen in liquid nitrogen and stored at -70°C.

Homogenization and Extraction

1. Samples of animals injected with radioactive tyrosine derivatives and stored in test tubes at -70°C were loosened from the test tube wall with a cold, dry spatula and inserted into glass cone grinders. To extract small molecules, each sample was ground with 0.3 ml of a solution of 10% acetic acid, 40% methanol, and 50% water. The samples were centrifuged in a clinical centrifuge. The supernatant was collected with a fine capillary tube. The residue was reground with 0.2 ml of grinding solution, centrifuged, and the supernatant collected and pooled with the first supernatant.

2. Extraction of some larger peptides and proteins was effected by grinding the residue of the first step with 0.5 ml of 0.1 M NaOH and allowing the solution to stand for two hours. The 0.1 M NaOH solution was centrifuged; the supernatant was collected with a fine glass capillary; and the residue was saved.

Washing and Homogenization of Pupal Cases

Pupal cases, usually in lots of 50 mg, were ground in water in a glass cone grinder by a motor driven pestle. The residue from centrifugation in a clinical centrifuge was

washed two times with 50% methanol at 70°C, two times with a detergent solution at 100°C, and two times with unheated distilled water.

Acid Hydrolysis of Pupal Cases

Following generally the method of Andersen (38), the homogenized and washed pupal cases were refluxed in 100 ml of 1N HCl for 3 hours or 100 ml of 6N HCl for 12 hours. The acid solution was centrifuged at 10,000 xg in a Servall SS-34 head for 15 minutes. The supernatant was evaporated to dryness in a rotary evaporator and redissolved in 0.2 M acetic acid.

Enzyme Hydrolysis of Pupal Cases

The Calbiochem chitinase was prepared by centrifuging a 1 mg/ml solution of chitinase in water at 28 x 10^3 xg in a Servall SM-24 head to eliminate debris. 50 mg portions of pupal case preparations were first incubated with 3 mg of chitinase in a 30 ml solution of 0.02 M phosphate buffer pH 5.0 containing 0.25 mg of sodium penicillin G at 34°C for 12 hours. The pH of the solution was changed to pH 6.8 with the addition of 1 M K_2HPO_4, and 2 mg of pronase were added. The incubation was continued for 24 hours. After incubation the solution was centrifuged for 15 minutes at 10,000 xg in

a Servall SS-34 head. The supernatant was lyophilized or evaporated to dryness in a rotary evaporator. After evaporation the residue was taken up in 1.5 ml of 0.2 N acetic acid and put over a P-2 column eluted with 0.2 N acetic acid for desalting. After desalting the sample was brought to dryness by lyophilization or rotary evaporation.

Paper Chromatography

Ascending paper chromatography on sheets of filter paper 25 cm x 15 cm was used to separate common tyrosine derivatives. A solvent of 66% propanol, 33% water, and 1% acetic acid was found to give good separation.

Column Chromatography

Gel chromatography Bio-Gel P-2 (Biorad Laboratories) and Sephadex G-15 fine and G-100 fine (Pharmacia Fine Chemicals) were swollen overnight in distilled water and washed several times by settling in distilled water. Void volumes were determined with Blue Dextran (Pharmacia Fine Chemicals). For the P-2 and G-15 columns elution was effected with 0.2 M acetic acid; for the G-100 columns elution was effected with 0.02 M phosphate buffer pH 6.8.

Cellulose phosphate Cellulose phosphate (Whatman P-11) was washed extensively with 1.0 HCl. Elution of the column (25 cm x 2 cm) was effected by an NaCl gradient established

by running 1000 ml of 1 M NaCl in 0.2 M acetic acid into a mixing flask containing 1000 ml of 0.2 M acetic acid. The column was run at room temperature under a pressure head of 20 cm, and 1 ml fractions were collected.

High Voltage Paper Electrophoresis

The 10,000 volt Gilson electrophorator was used for high voltage paper electrophoresis. The method was that described by Dreyer and Bynum (67).

Acrylamide Gel Electrophoresis

Acrylamide gel electrophoresis in SDS was performed by the method of Wever and Osborn (68). Gels were 10% acrylamide and 0.14% bis-acrylamide. Gels were run at 6 ma/tube. Gels were stained in 0.25% Comassie Brilliant Blue R-250 (Mann Research Laboratories) in methanol: acetic acid: water, 5:1:5 by volume, for 24 hours. They were destained by diffusion in 5% methanol and 10% acetic acid.

Samples and standards were prepared by heating at 100°C for 20 minutes in 0.1% (w/v) SDS, 0.1% (w/v) beta-metcaptoethanol, and in 0.1 M sodium phosphate, pH 7.0. Standard proteins were myoglobin (A grade, Calbiochem), BSA (crystallized, Sigma), and Catalase (C-100, Sigma).

Color Reagents

Ninhydrin in acetone (1% w/v) or a cadmium-ninhydrin reagent (67) was used to locate amino acids by dipping paper chromatography paper or high voltage electrophoresis paper into the ninhydrin solution. A ferric chloride-potassium ferrocyanide reagent (38) was used to localize phenolic compounds on paper by dipping.

Radioactive Counting

Soluble ^{14}C or ^{3}H labelled material in aqueous media was counted in a PPO/Naphthalene/Dioxane (4g:100g:1L) scintillation fluid.

Material insoluble in aqueous media - in these experiments consisting mostly of insoluble cuticle or pupal case residue - was ground as finely as possible and suspended in an aquasol (New England Nuclear) gel (77% Aquasol/23% water) for scintillation counting.

Relative counting efficiency between the dioxane method for counting soluble labelled material and the Aquasol method for counting insoluble material was determined by the following method: a sample of finely homogenized ^{14}C labelled pupal cases was divided into two equal lots. One lot was completely hydrolyzed by refluxing in 6N HCl; the HCl was removed by rotary evaporation and the sample dissolved in water. The other untreated lot was suspended in water. The soluble

sample was counted in dioxane scintillation fluid, and the insoluble sample was counted in the Aquasol gel. Counting efficiency for the Aquasol method was found to be 10% to 15% lower than for the dioxane method using ^{14}C label. Tritium label was not used in experiments in which counting of label in insoluble material was involved.

To localize spots of radioactive material on chromatography paper, the part of the paper on which the sample moved was cut into strips 0.5 cm or 1 cm wide and counted with dioxane scintillation fluid. The counting efficiency for ^{14}C label on chromatography paper was found to be within 5% of the efficiency for soluble ^{14}C label.

Counting was done in a Beckman LS-200 scintillation counter at a gain of 350 in a pre-set H^3-C^{14} window. Soluble counts and counts on paper are reported without adjustment except for subtracting the background of 40 cpm. The insoluble counts are adjusted to make the counting efficiency equal to that of the soluble counts.

Internal Pressure Measurement

Internal pressure of larvae, prepupae, and pupae was measured by means of an apparatus consisting of a needle with a fine, beveled point (the same type of needle that is used for injection), a mercury column, and a hand held rubber bulb for applying pressure. The three components are interconnected

with Pe-20 tubing. The needle is inserted into the animal between two segments of the posterior part just beneath the cuticle, where it remains while the pressure is being measured. The principle of the method is to counter-balance the internal pressure of the animal by the pressure applied by means of the rubber bulb. Too great an applied pressure forces air bubbles into the animal; applied pressure less than internal pressure allows hemolymph to rise in the needle. The disadvantage of the method is that it is not accurate at internal pressures less than 10 mm Hg. With animals that appear to have no internal pressure, i.e., the hemolymph - air meniscus does not rise in the needle, an external pressure of about 10 mm Hg is necessary to force the hemolymph-air meniscus to move. This inertia, which is not present when the needle is inserted into water instead of into the animal, is possibly due to the greater viscosity of the hemolymph. The opening in the end of the needle is too large for capillarity to be a factor. The volume of the part of the needle that is inside the animal is too small to increase the pressure enough to affect the 10 mm error already present.

RESULTS

^{14}C Tyrosine injection

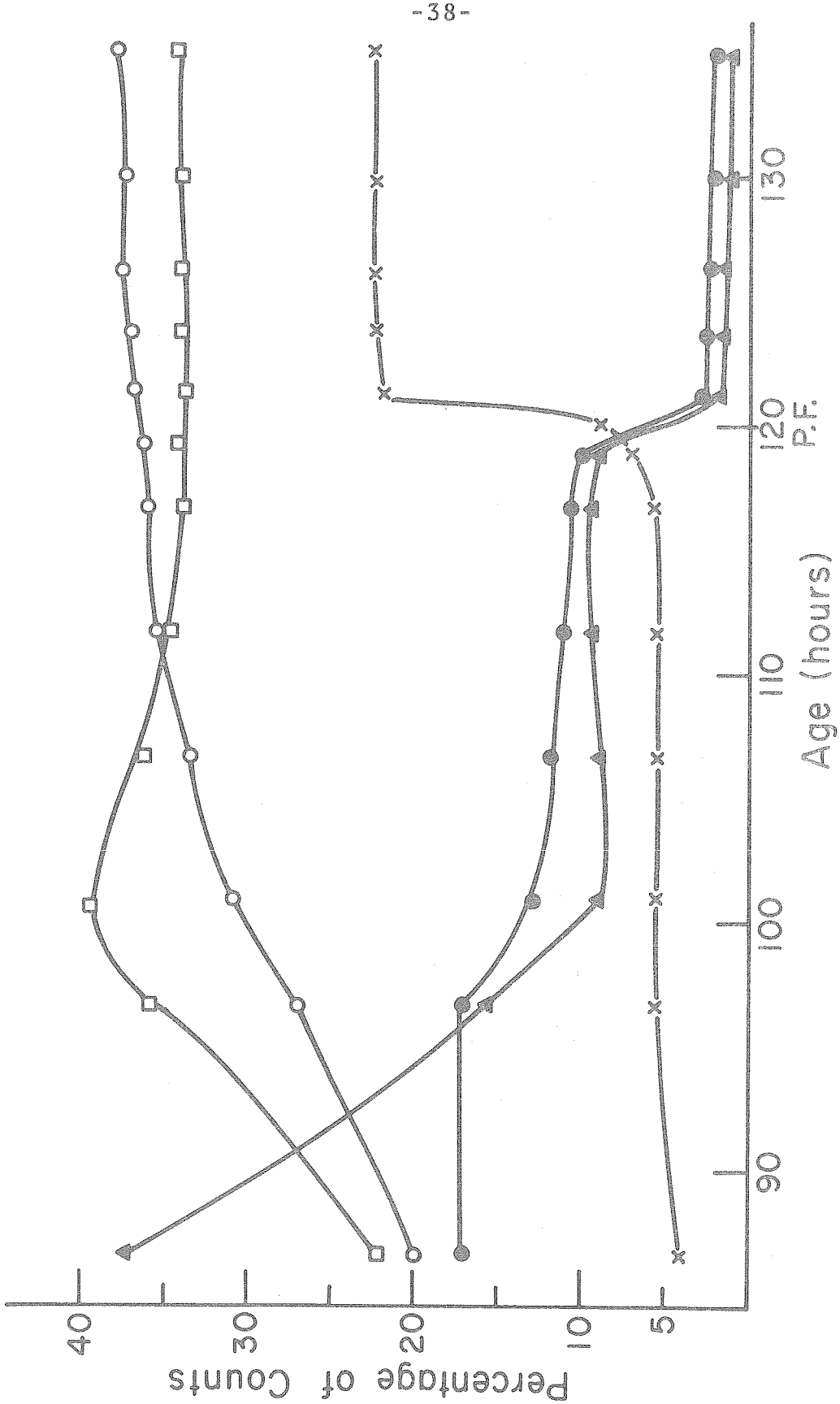
The fate of ^{14}C tyrosine was followed from the third instar larval stage through puparium formation and beyond. The experiments were initiated by injecting ^{14}C (U) tyrosine into at least 100 third instar larvae all the same age within about 6 hours. The wild type was generally used. In some experiments ebony was used; however, the results were essentially the same as for wild type. After injection the larvae were returned to their food and samples of about ten animals were taken at various times before and after puparium formation. Since events around puparium formation were most interesting, more samples were taken during this interval than at other times. The label from each sample was counted in four fractions: (1) small molecules that are extracted by an extraction solution of 50% methanol: 40% water: 10% acetic acid and that move from the origin when chromatographed on paper, (2) peptides which are extracted but which remain at the origin when chromatographed on paper, (3) large peptides and proteins that are extracted with 0.1N sodium hydroxide, (4) material - mostly cuticle material - that is not extracted with 0.1N sodium hydroxide.

Results of Experiment 1, in which 80 hour larvae were

injected, is shown in Figure 1. The third instar begins at 72 hours and lasts until puparium formation at 120 hours; tanning and hardening of the puparium lasts for about 3 hours after puparium formation. From Figure 1, it is seen that equilibrium of the label for the larval period among the four fractions is almost reached around 95 hours. At puparium formation the number of counts in Fraction 1 decreases to about the same extent that the number in Fraction 4 increases. There appears to be no change before and after puparium formation in the number of counts in Fraction 2 and Fraction 3. Of the tyrosine metabolites that move from the origin of the paper during paper chromatography significant amounts of label were found only in tyrosine and tyrosine-O-phosphate.

To verify that Fraction 4 consists mostly of cuticle or pupal case material, the proportion of ^{14}C ketocatechol, which can be extracted only from the hardened puparium by methods to be described in a later section in this thesis, to total counts was compared between Fraction 4 and shed pupal cases that had been extracted with 0.1N sodium hydroxide. The larvae in the experiment were injected at 110 hours. For the shed pupal cases, 72% of the counts were ^{14}C ketocatechol; for Fraction 4 from animals about 6 hours after puparium formation, 60% of the counts were ^{14}C ketocatechol. These results show that over 80% of Fraction 4 is pupal case material.

Figure 1. ^{14}C(U) Tyrosine injection into 80 hour larvae. Approximately 170 larvae were initially injected. Samples of 10 animals were taken at each time shown in the figure. Animals were collected, stored, homogenized, and fractionated as described in Materials and Methods. Each point represents the percentage of the total counts in the sample in that fraction. Each sample contained approximately 5×10^4 cpm. Solid circles represent tyrosine; triangles, tyrosine-O-phosphate; crosses, 0.1N NaOH insoluble material; circles, 0.1N NaOH soluble material; squares, material that remained at the chromatographic origin.


Percentage of Counts
40
30
20
10
5
90
100
110
120
130
P.F.
Age (hours)

In Figure 2 the results of Experiment 2, in which the larvae were injected at 107 hours of age, are shown. Protein synthesis appears to slow down around puparium formation as very little label is incorporated into Fraction 3 as compared to the results of Experiment 1. About the same proportion of counts is present in Fraction 2 of both Experiments 1 and 2. Just before puparium formation it can be seen that some tyrosine-O-phosphate counts are lost to the pool of free tyrosine; all tyrosine-O-phosphate is probably converted to tyrosine but a build-up of tyrosine is not seen because of the rapid incorporation of the tyrosine into the puparium. The proportion of label in the pool of free tyrosine and tyrosine-O-phosphate remains high until puparium formation when, as in Experiment 1, there is an abrupt drop. Again, Fraction 4 increases to about the same extent that Fraction 1 decreases, indicating a net flow of tyrosine into the puparium from Fraction 1. The low level of Fraction 1 after puparium formation as compared to the high level before indicates that almost all the pool of free tyrosine and tyrosine-O-phosphate is lost to the puparium.

In Figure 3 are results from Experiment 3, in which larvae were injected at 97 hours. In this case, the label that is incorporated into the puparium is not all accounted for by a decrease in the pool of free tyrosine and tyrosine-O-phosphate. Fraction 2 and 3, also, appear to lose counts

Figure 2. ^{14}C Tyrosine injection into 107 hour larvae. This experiment was done by the same method as described in Figure 1. Each sample contained approximately 7.0×10^4 cpm. Symbols are the same as in Figure 1.

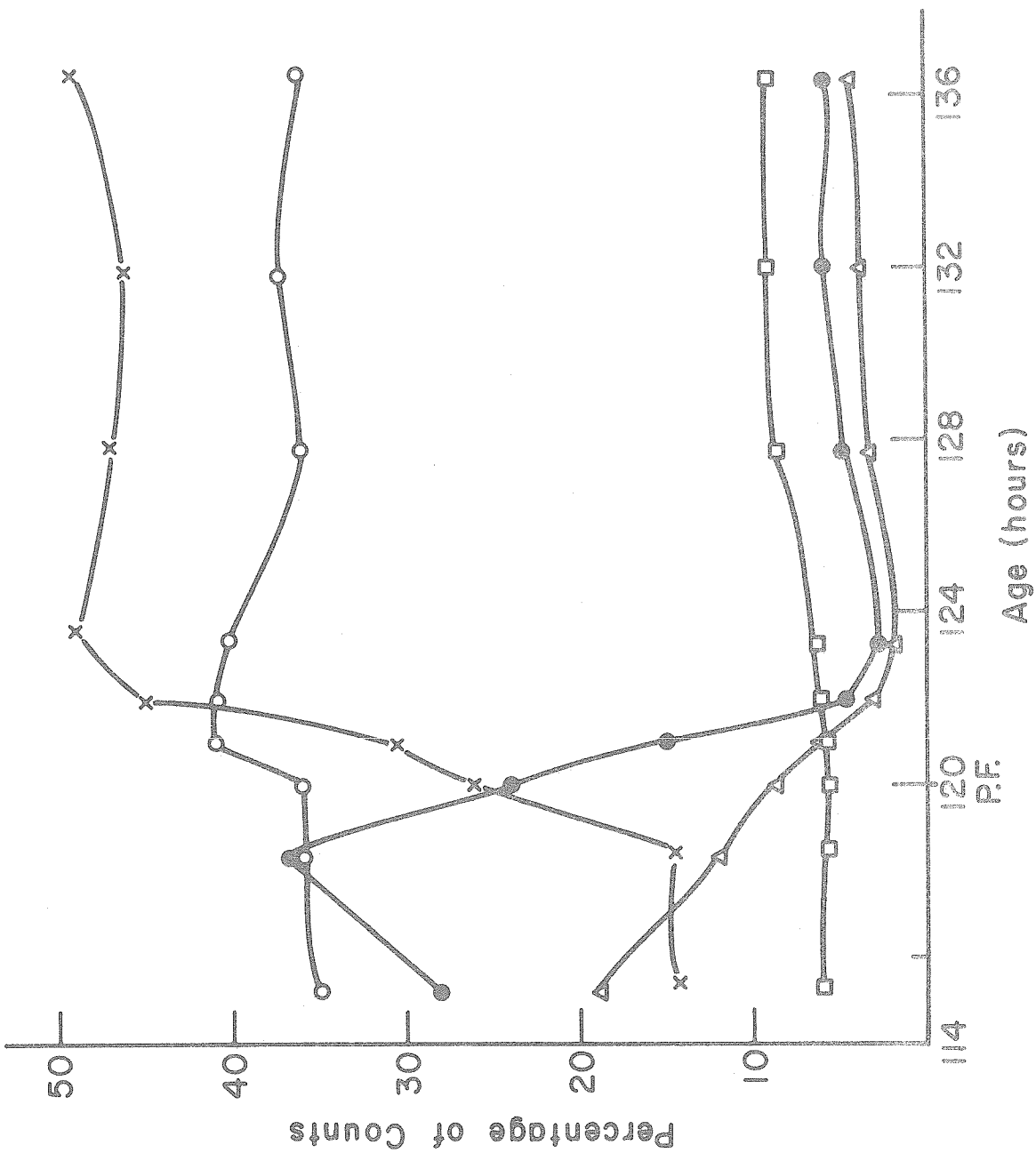
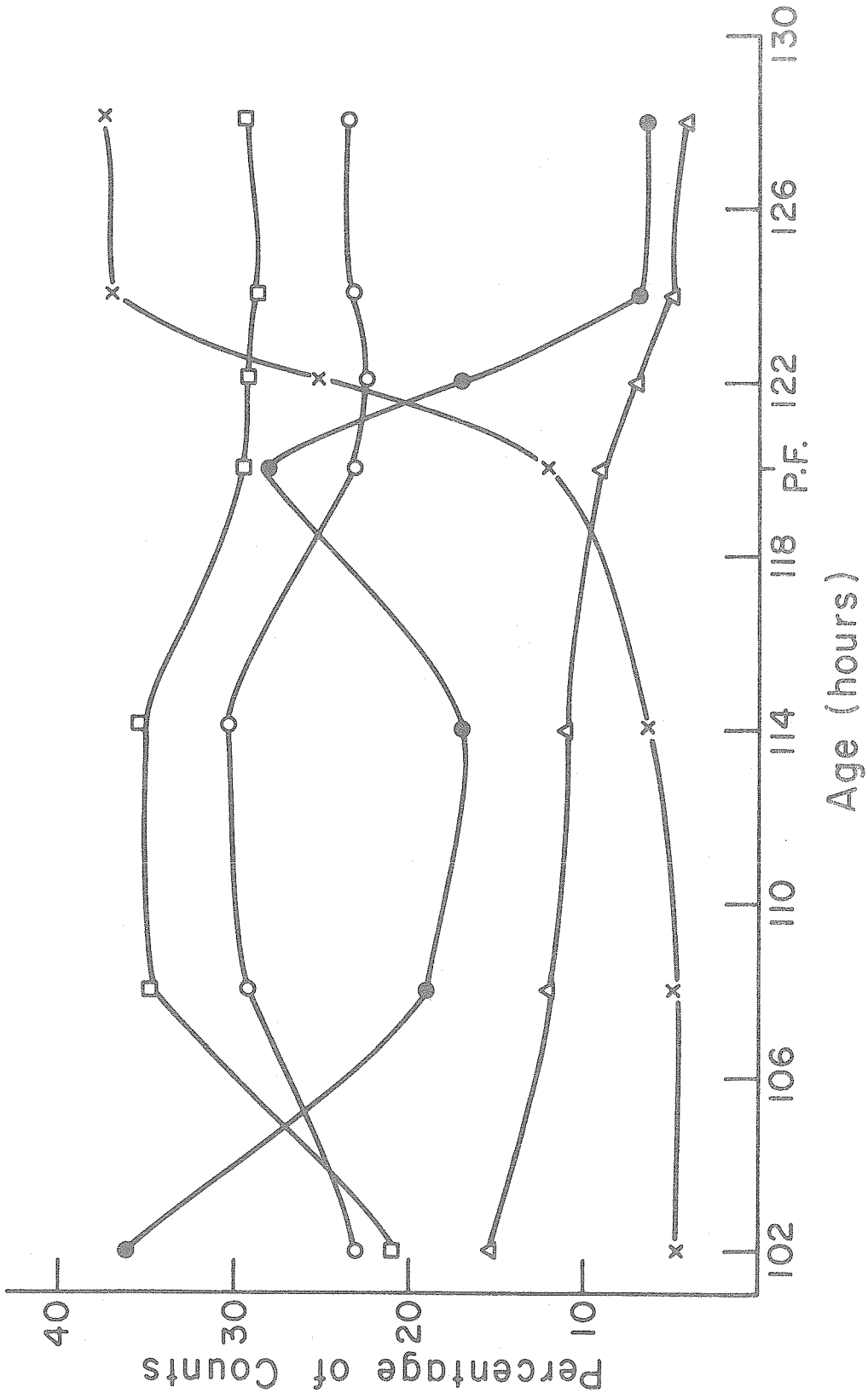

Percentage of Counts
50
40
30
20
10
4
1
20
P.F.
24
128
1
32
136
Age (hours)

Figure 3. ^{14}C Tyrosine injection into 97 hour larvae. This experiment was done by the same method as described in Figure 1. Each sample contained approximately 1.0×10^4 cpm. Symbols are the same as in Figure 1.


Percentage of Counts
40
30
20
10
102
106
110
114
118
P.F.
122
126
130
Age (hours)

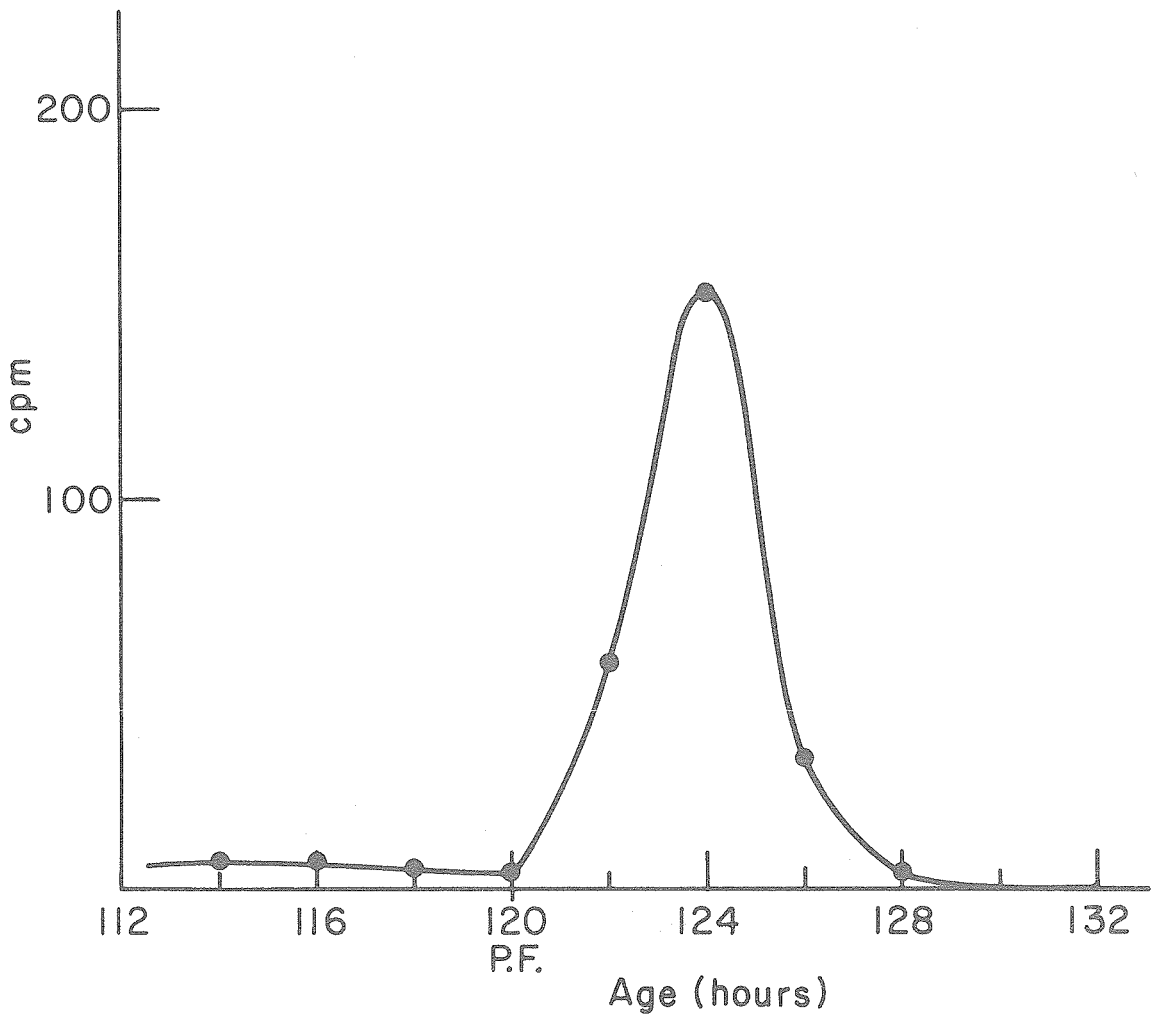
at puparium formation to Fraction 4. There is a rise in the levels of tyrosine at puparium formation that is not accounted for by hydrolysis of tyrosine-O-phosphate.

Results such as in Figure 3 were rare among the numerous experiments involving ^{14}C tyrosine injection into different age groups of third instar larvae. Usually, regardless of time of injection, these experiments gave results similar to those of Experiments 1 and 2 in that the label incorporated into the puparium appeared to be derived exclusively from the pool of free tyrosine and tyrosine-O-phosphate.

In some of the ^{14}C tyrosine injection experiments a very small peak appeared on paper chromatography at R_f 0.92, the R_f of the standard N-acetyldopamine with a chromatography solution of 66% propanol: 33% water: 1% acetic acid. In Figure 4, what appears to be N-acetyldopamine becomes detectable for a few hours after puparium formation, possibly indicating that the turnover of N-acetyldopamine is not so rapid as the sites at which it is incorporated in the puparium begin to become filled. A high number of counts injected into the larvae was necessary so that the peak could be distinguished from background counts. The appearance of N-acetyldopamine did not seem to depend on the age of the larvae when injected.

In summary, most of the data from the ^{14}C tyrosine injection experiments suggest that during the time of puparium

Figure 4. N-acetyldopamine peak. The method was the same as described in Figure 1. The larvae were injected at 100 hours. Each sample contained approximately 5.0×10^4 cpm. The vertical axis is cpm and the horizontal axis is larval age.


200
100
cpm
112
116
120
P.F.
124
128
132
Age (hours)

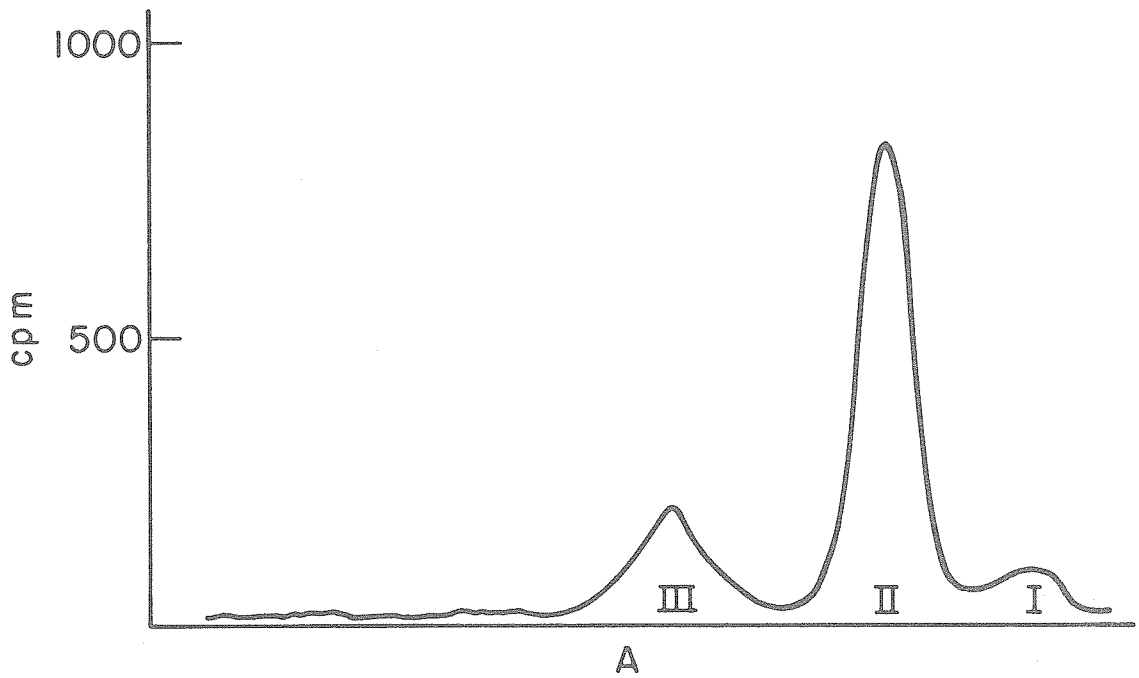
formation the pool of free tyrosine and tyrosine-0-phosphate - which at that time is hydrolized to tyrosine - is almost entirely incorporated into the puparium. The sclerotizing agent, if other than tyrosine, was not detected in these experiments before puparium formation, indicating that incorporation of the sclerotizing agent must be very rapid after its synthesis. The appearance of what appeared to be N-acetyldopamine toward the end of the hardening and tanning period is interesting in view of the fact that it has been established as the sclerotizing agent of Calliphora.

Injection of labelled tyrosine metabolites

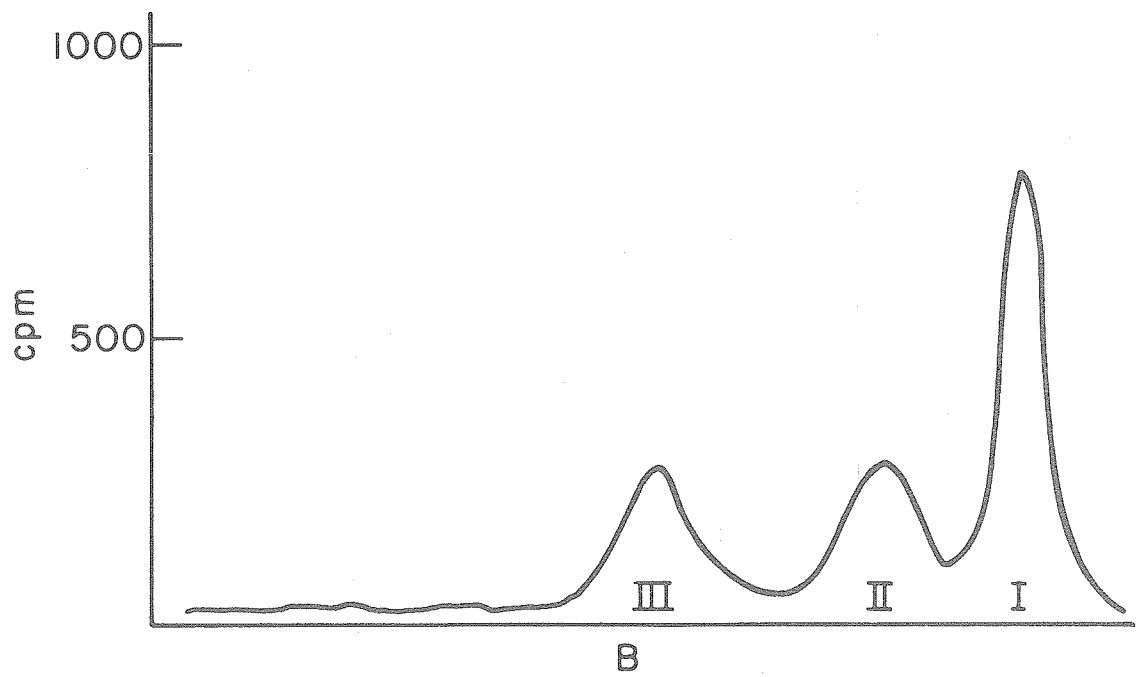
Tyrosine-0-phosphate ^{14}C (U) Tyrosine-0-phosphate (380 mCi/mmole), synthesized as described in Materials and Methods, was injected into third instar larvae of various ages. At certain times after injection, samples of 3 or 4 animals were taken and either squashed directly on paper for paper chromatography or extracted with a solution of 50% methanol: 40% water: 10% acetic acid and chromatographed on paper.

In Figure 5 the proportion of label among tyrosine, tyrosine-0-phosphate, and material that remains at the origin of the paper chromatogram is shown at 25 minutes and at 6 hours after injection of 90 hour larvae. At 25 minutes after injection of ^{14}C tyrosine-0-phosphate, ^{14}C tyrosine has already

Figure 5. ^{14}C(U) Tyrosine-0-phosphate (380mCi/mmole) injected into 90 hour larvae. Samples of 3 animals were squashed directly on the chromatograms, which were developed with 66% n-pro-panol: 33% water: 1% acetic acid. After development the chromatograms were scanned by the Actigraph II with a gas flow, micromil end window Geiger tube (Nuclear-Chicago). Scannings of a sample squashed at 25 minutes (A) and a sample squashed at 6 hours after injection (B) are shown. Peak 1 is origin material; Peak 2 is tyrosine-0-phosphate; Peak 3 is tyrosine.



A



B

appeared. No other peaks of radioactivity are present other than a comparatively small number of counts at the origin. At 6 hours after injection, there are no additional peaks of radioactivity. The injected tyrosine-0-phosphate label has come to an equilibrium state that will remain until puparium formation. At 6 hours after injection, the tracing of the chromatogram is the same whether ^{14}C tyrosine or ^{14}C tyrosine-0-phosphate is injected.

Data from ^{14}C tyrosine-0-phosphate injection experiments with larvae injected a few hours before puparium formation confirm the idea that tyrosine-0-phosphate is converted to tyrosine before being incorporated into the puparium. At times just before puparium formation, the tyrosine-0-phosphate to tyrosine reaction is much more rapid than at other times; almost all the counts are present as ^{14}C tyrosine 30 minutes after injection.

Incorporation of ^{14}C tyrosine-0-phosphate into unusual products was looked for. No substances that migrate during paper chromatography or high voltage paper electrophoresis other than tyrosine, tyrosine-0-phosphate, and a low background of peptides was found at any time after injection. The identity of the substances at the origin of the paper chromatogram into which label is incorporated is not known. Certainly, as ^{14}C tyrosine begins to build up after injection of ^{14}C tyrosine-0-phosphate, the origin material contains

many products of tyrosine incorporation such as peptides and proteins. Seemingly, the best time to look for tyrosine-O-phosphate incorporation products at the chromatogram origin is just after injection before tyrosine and its incorporation products build up. In fact, the accumulation of free tyrosine always preceded accumulation of significant counts at the origin, indicating that labelled substances at the origin even soon after injection of ^{14}C tyrosine-O-phosphate are mostly products of ^{14}C tyrosine incorporation.

Dopa 3,4-Dihydroxyphenylalanine-2,3- ^{3}H (1 Ci/mmole) was injected into third instar larvae of various ages. Samples of 2, 3, or 4 animals were taken after injection and treated by the same method as used in the ^{14}C tyrosine-O-phosphate experiments. In order not to upset the metabolism of the organism and, perhaps, derive spurious results, the amount of radioactive substance injected should be small compared to the endogenous pool of that substrance. The amount of dopa per late third instar larva has been found to be 1.5 ± 0.3 ng (70) or 2.0×10^{3} times less than the quantity of tyrosine and tyrosine-O-phosphate per late third instar larva. In most experiments, ^{3}H dopa was injected in a solution of 50 microCi/ml; about 0.04 microliter of the solution is injected into each animal. The quantity of ^{3}H dopa injected was at most about 20% of the endogenous pool of dopa. It was noted that counts were lost from injected

larvae; the loss of counts was especially marked when ^{3}H dopa solutions of more than 50 microCi/ml were injected. Evidently, the size of the dopa pool is critical, and any excess dopa is broken down.

The fate of ^{3}H dopa 30 minutes after injection using late third instar larvae is shown in Figure 6. With the 66% n-propanol: 33% water: 1% acetic acid paper chromatography system, dopa has an R_f of 0.4, dopamine an R_f of 0.7, and N-acetyldopamine an R_f of 0.9. Peaks corresponding to dopa, dopamine, and N-acetyldopamine are present as well as a large peak at the origin. The turnover of ^{3}H dopa is rapid: after 2 or 3 hours almost all the injected counts have either been lost from the animal or incorporated into material at the origin. The counts at the origin are certainly at least partly incorporated into cuticle material: counts are recovered when shed pupal cases from animals injected with ^{3}H dopa are solubilized in boiling 1N HCl. Because of the difficulty of injecting the same amount into different larvae and because of the loss of counts after injection, it is not known whether the counts incorporated into material at the origin are entirely recovered from the shed puparia.

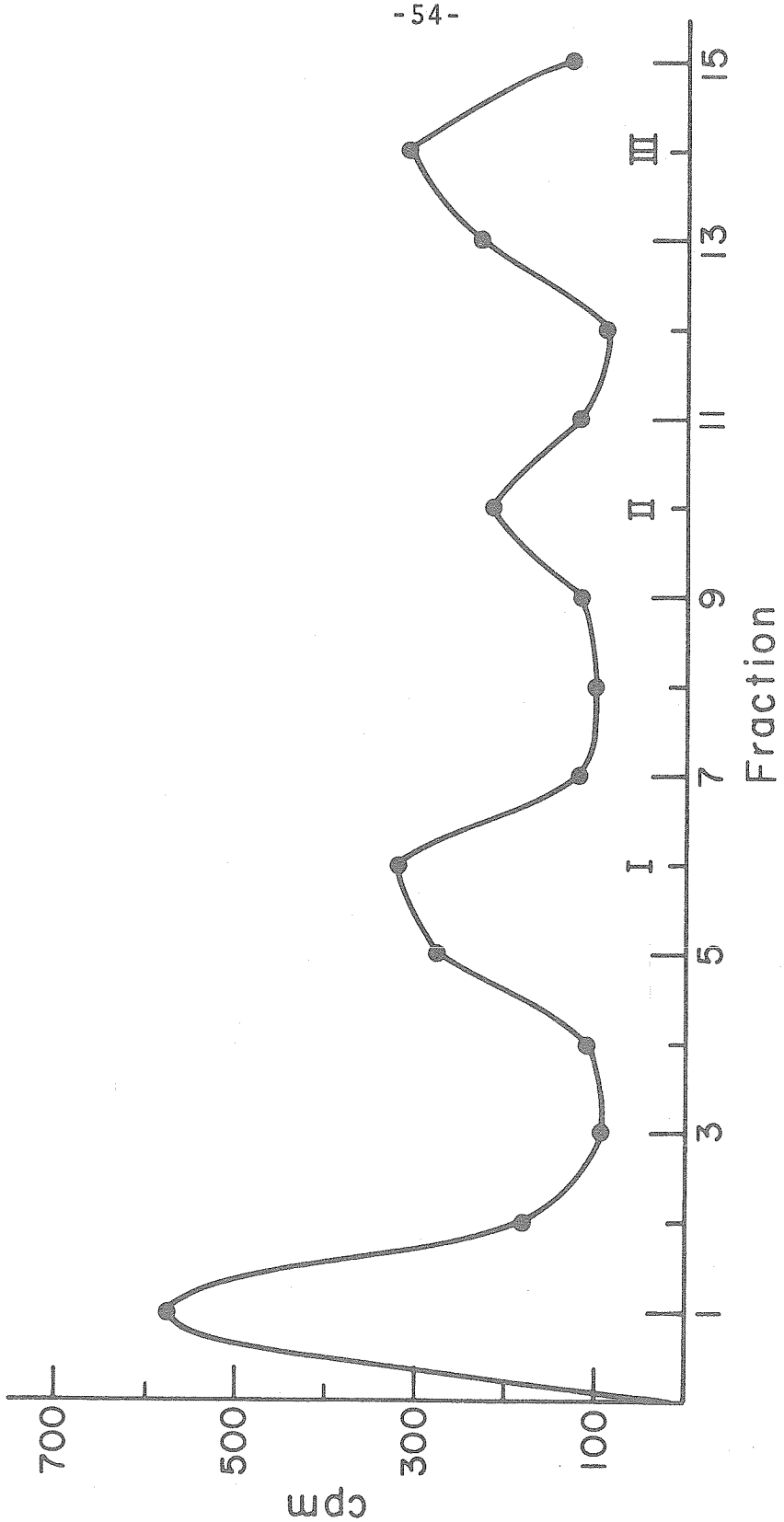
Dopamine Dihydroxyphenylethylamine-2-^{3}H (5Ci/mmole) was injected into late third instar larvae in a solution of 40 microCi/ml. Late third instar larvae contain less than 0.1 ng per larva. ^{3}H dopamine of an amount at least equal

Figure 6. 3,4-Dihydroxyphenylalanine-2,3-^{3}H (1Ci/mmole) injected into 105 hour larvae. At 30 minutes after injection, 4 larvae were squashed directly on the paper chromatogram, which was developed with a solution of 66% n-propanol: 33% water: 1% acetic acid. After development the chromatogram was cut into 1 cm strips, which were counted in dioxane counting solution.

1 Dopa

2 Dopamine

3 N-acetyldopamine


700
500
300
100
cpm
1
3
5
7
9
11
13
15
I
II
III
Fraction

to the amount of endogenous dopamine was injected.

At 20 minutes after injection of late third instar larvae, the ^{3}H dopamine is incorporated into material at the origin and converted into N-acetyldopamine and, to a small extent, into two unknown substances. Possibly, the 2 smaller peaks represent breakdown products of dopamine, since counts are lost from ^{3}H dopamine injected larvae. The identity of the substance with an R_f of 0.9 which was taken to be N-acetyldopamine was more surely established by the finding that most of the methanol extracted counts from ^{3}H dopamine injected larvae are either dopamine or N-acetyldopamine as determined by high voltage paper electrophoresis using dopamine and N-acetyldopamine standards.

N-acetyldopamine N-acetyldopamine-2-^{3}H (5 Ci/mmole) was synthesized as described in Materials and Methods and injected into late third instar larvae in a solution of 40 microCi/ml. The radioactivity profile from an ^{3}H N-acetyldopamine injection (Figure 8) is much like that from an ^{3}H dopamine injection. Within 2 or 3 hours of injection most of the label has been incorporated into material that remains at the origin of the chromatogram. Counts are recovered from the shed pupal cases of injected larvae. It is interesting that some ^{3}H N-acetyldopamine is converted to dopamine; it might be expected that, if N-acetyldopamine is the sclerotizing agent, the dopamine to N-acetyldopamine

Figure 7. Dihydroxyphenylethylamine-2-^{3}H(5Ci/mmole) injected into 105 hour larvae. At 20 minutes after injection, 4 larvae were squashed directly on paper. The rest of the procedure is described in Figure 6.

2 Dopamine

3 N-Acetyldopamine

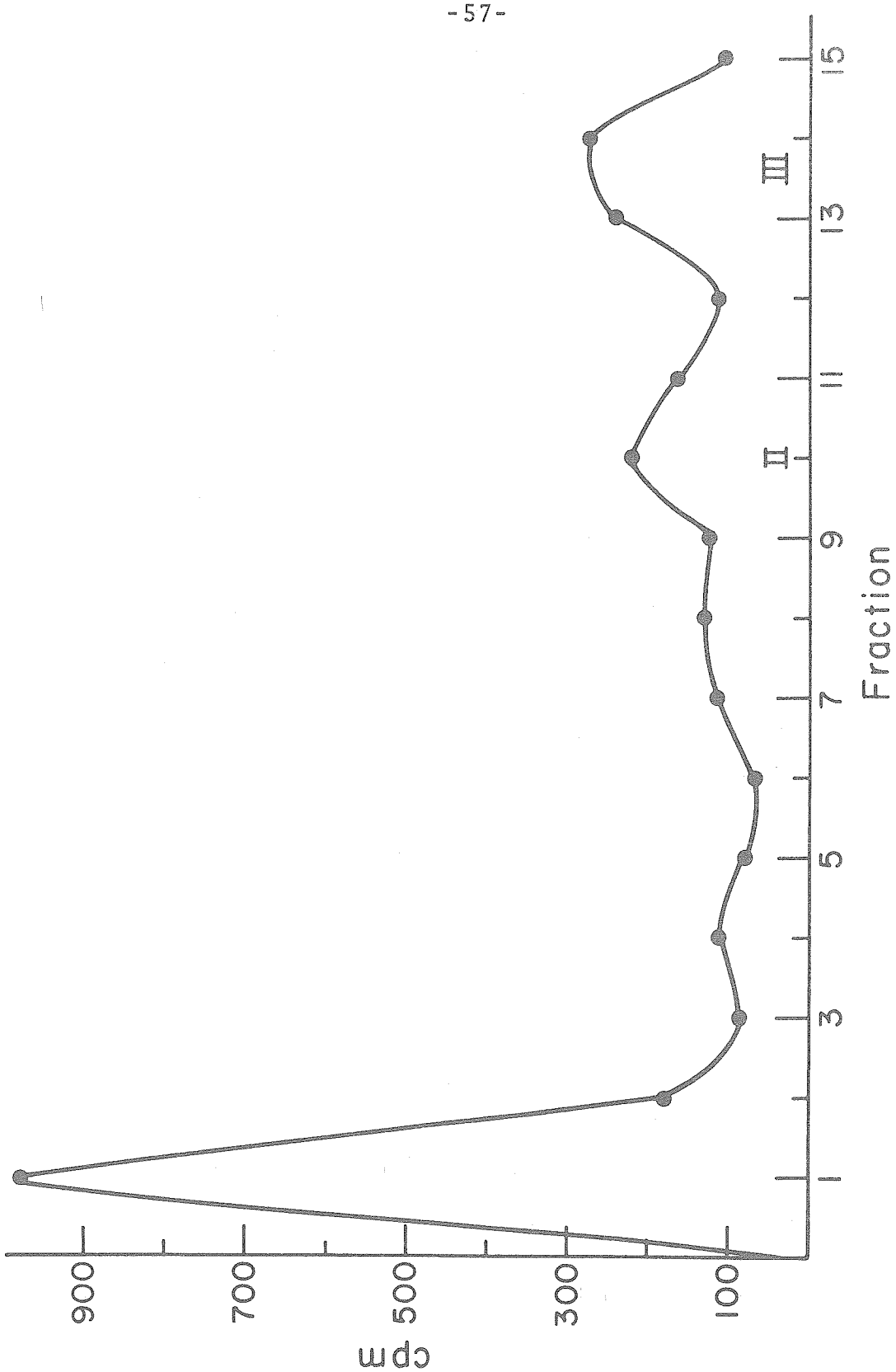
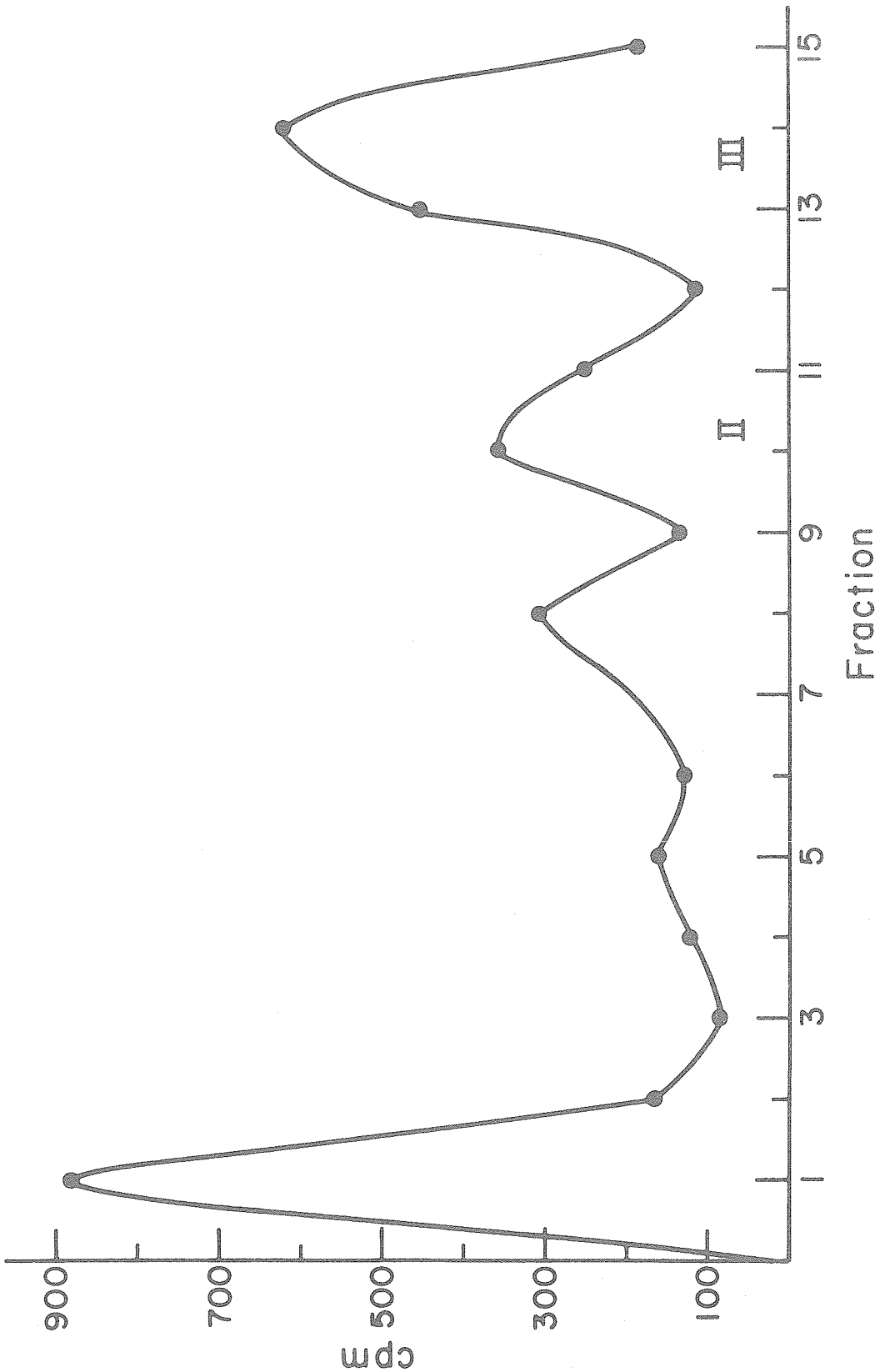

cpm
600
700
500
300
100
1
3
5
7
9
II
11
13
III
15
Fraction

Figure 8. N-acetyldopamine -2-^{3}H(5Ci/mmole) injected into 105 hour larvae. At 20 minutes after injection 4 larvae were squashed directly on paper. The rest of the procedure is described in Figure 6.

2 Dopamine

3 N-Acetyldopamine


900
700
500
300
100
cpm
1
3
5
7
9
11
13
15
Fraction
II
III

reaction would be one way. The appearance of dopamine could, also, be an artifact due to injecting more N-acetyl-dopamine than is normally present in the larva.

Late Third Instar Larval and Early Prepupal Protein

Proteins were examined before and after puparium formation to find if there is any significant decrease in any fraction. Such a decrease might indicate involvement of that fraction in storing tyrosine for incorporation into the puparium at puparium formation. Some work bearing on this has been done: Boyd (71) found little difference in the pattern of hemolymphal proteins on acrylamide gel between late larvae and early pupae. Winicur (72) showed that the total soluble protein decreases by about 15% after puparium formation. Mitchell and Simmons (73, 74) found no tyrosine rich peptide in their investigations of peptides of Drosophila.

Soluble proteins of late third instar larvae and prepupae soon after tanning and sclerotization were extracted with electrophoresis buffer to which phenylthiourea was added to block phenoloxidase activity and run on acrylamide gels at less than 4°C. By visual inspection, the way that the proteins vary with respect to each other is almost identical for the two ages.

The technique of SDS extraction and SDS acrylamide gel electrophoresis was used to examine proteins which are not soluble in buffer at less than 4°C. The method was that

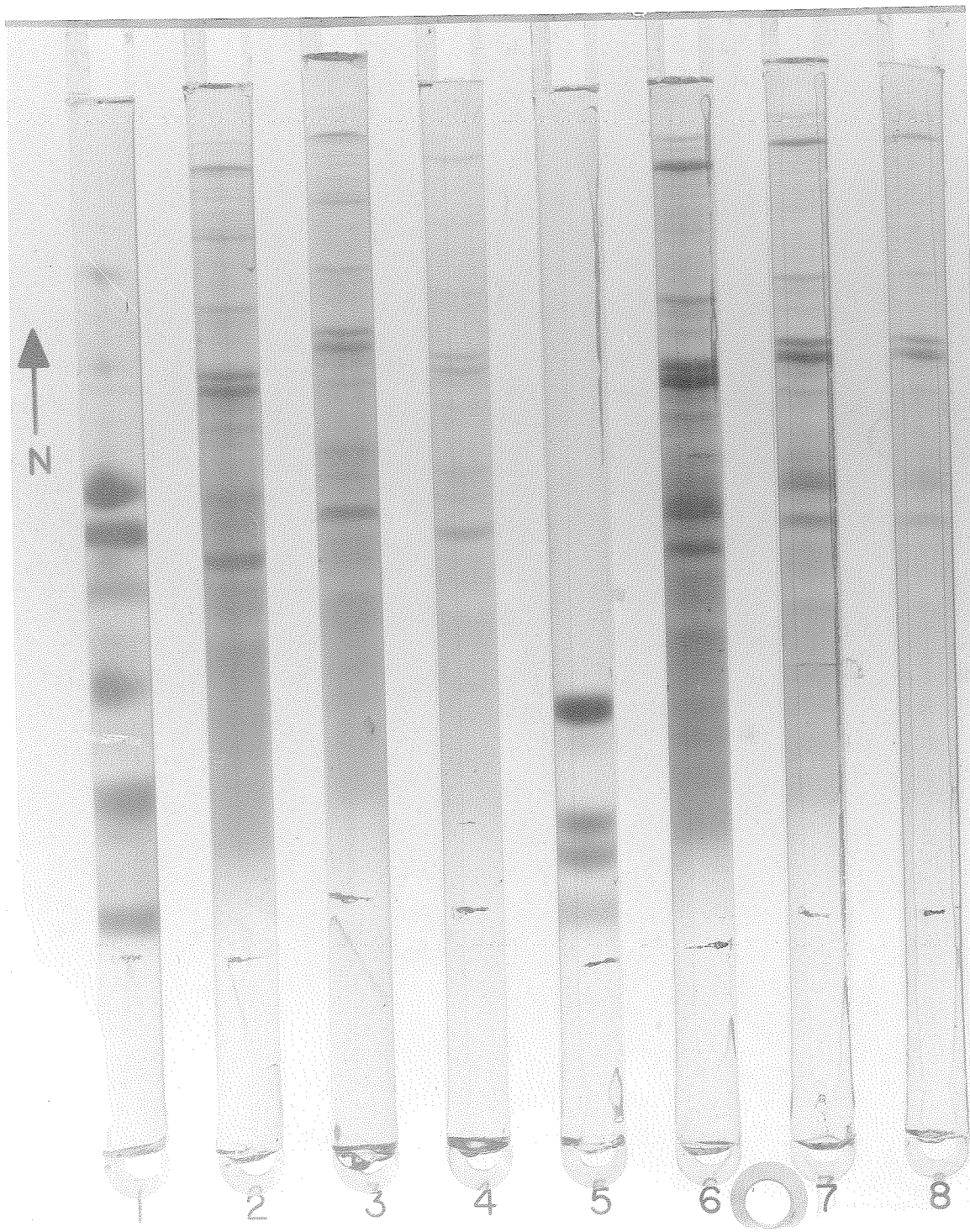
described in Materials and Methods and was carried out by Dr. John Smart. The SDS gel electrophoresis patterns for late third instar larvae and early prepupae may be compared in Figure 9. There are differences in the way that the proteins vary with respect to each other. The total amount of proteins shown at the two different ages cannot be compared.

Products of Acidic Degradation of Pupal Cases

Andersen's finding (37, 38, 39) that ketocatechols in relatively large quantities can be extracted by acid from hardened locust cuticle led to an investigation to determine what phenol products can be extracted from Drosophila pupal cases. One aim of these experiments was to compare the extracted substances from the white pupal cases of ebony with the extracted substances from the tan pupal cases of wild type. Since the validity of such comparisons depends upon the accuracy of the data, most of the experiments described in this section have been often repeated. No significant difference in the quantity or type of the extracted phenol substances of ebony and wild type was found; thus, the values given apply to both.

Washed and homogenized pupal cases were initially extracted by refluxing in 1N HCl for 3 hours as described in Materials and Methods. That over 90% of the pupal case material is solubilized by this procedure was determined by com-

Figure 9. 10% SDS acrylamide gels. Material was first extracted with 0.02 M phosphate buffer pH 6.8; the SDS extract of the residue was run. Gels 2, 3, and 4 show the results of running different concentrations of 115 hour larval SDS extract. Gels 6, 7, and 8 are of extracts from 5 hour prepupae. Gel 1 contains the following standards in ascending order: (1) Insulin, 6000 Daltons; (2) hemoglobin, 15,500 Daltons; (3) gamma globulin (light chain), 23,000 Daltons; (4) pepsin, 35,500 Daltons; (5) ovalbumin, 43,000 Daltons; (6) gamma globulin (heavy chain), 50,000 Daltons.


N
1
2
3
4
5
6
7
8

parison of the absorbance at 280 Angstroms of the insoluble portion after hydrolysis by 6N HCl at 110°C for 5 hours with the absorbance of the soluble portion. Over 90% of the ^{14}C label from the pupal cases of animals injected with ^{14}C tyrosine in the third larval instar is solubilized by refluxing in 1N HCl.

Figure 10 shows the pattern of elution as determined by absorbance at 280 Angstroms when the 1N HCl extracted material is run over a P-2 column. Three main peaks were observed: the first peak was material excluded from the P-2 gel. The second peak was included material. The third peak was eluted after about two column volumes of elutant had passed through the column, indicating that the separation was not so much determined by gel-filtration as by reversible absorption to the polyacrylamide matrix of the P-2 gel.

The third peak was found to contain the same compound that Andersen exhaustively identified as a ketocatechol. The material from the third peak gave the shoulder at 310 Angstroms characteristic of ketocatechols. The UV spectra for the synthesized ketocatechol 2-hydroxy-3', 4' dihydroxy-acetophenone and the material in the third peak are very similar (Figure 11). The material appears to be homogeneous as judged by paper chromatography and high voltage paper electrophoresis. An R_f of 0.45 was observed in paper chromatography with the solvent system of benzene: acetic acid: water (125: 72:3); an R_f of 0.75 was observed with the solvent sytem of

Figure 10. Fractionation of a hydrolysate of Drosophila wild type puparia. Prior to hydrolysis the puparia were washed and homogenized as described in Materials and Methods. The dried material (50 mg) was hydrolysed by refluxing in 100 ml 1N HCl for 3 hours. The supernatant was evaporated to dryness, the residue redissolved in 1 ml of 0.2 N acetic acid and fractionated on a column of BioGel P-2 (25 cm x 2 cm^2). Elution was performed with 0.2 N acetic acid and the absorbance of the effluent at 280 Angstroms was recorded.

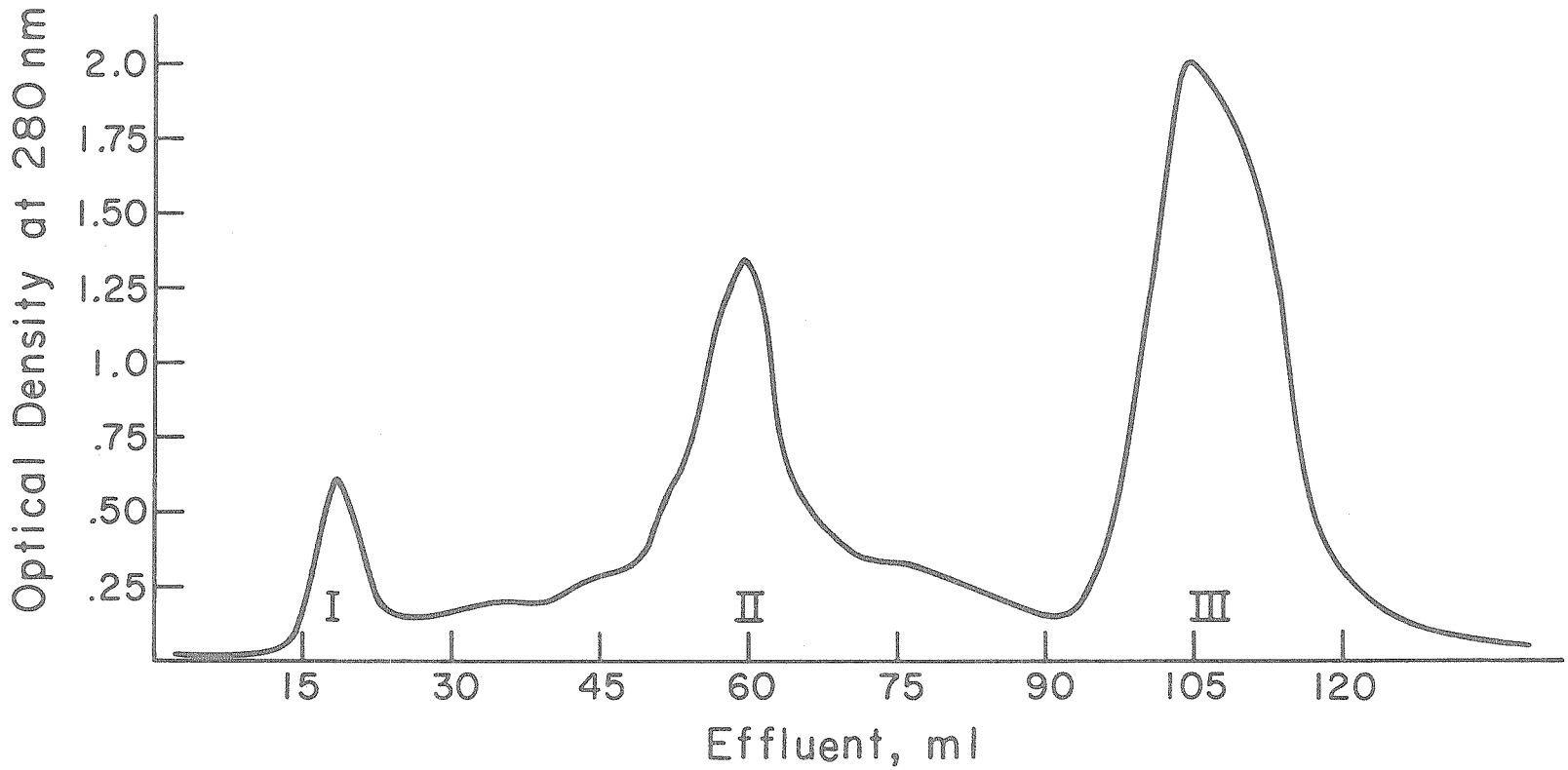
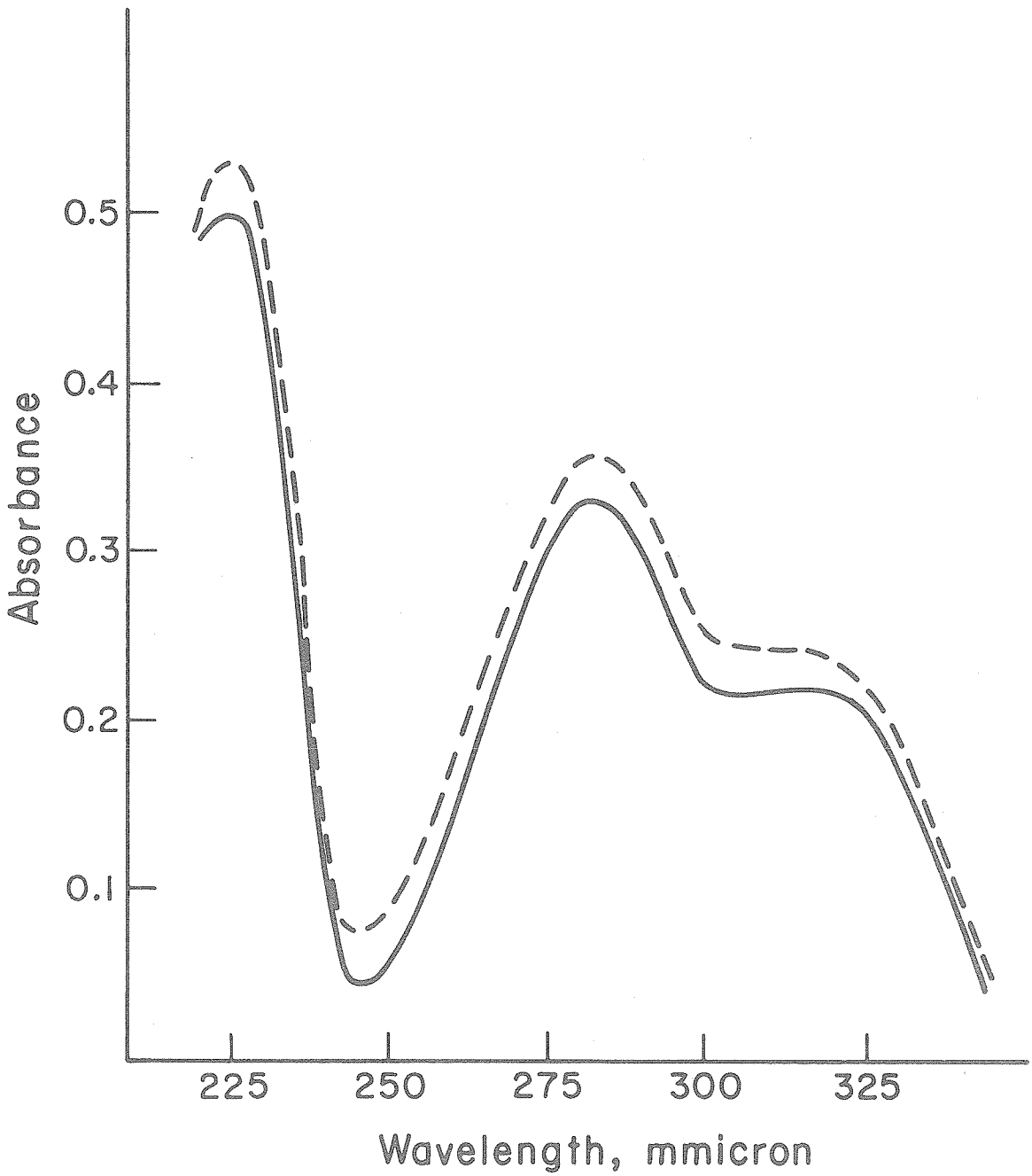

Optical Density at 280 nm
2.0
1.75
1.50
1.25
1.0
.75
.50
.25
I
II
III
15
30
45
60
75
90
105
120
Effluent, ml

Figure 11. UV spectra of 2-hydroxy-3',4' dihydroxyacetophenone (fully drawn curve) and material from the third peak of the P-2 column fractionation (broken curve). The substances were dissolved in 0.2 N acetic acid.


Absorbance
0.5
0.4
0.3
0.2
0.1
225
250
275
300
325
Wavelength, mmicron

n-butanol: acetic acid: water (3:1:1). The standard keto-catechol 2-hydroxy-3', 4' dihydroxyacetophenone chromatographed the same in both solvent systems as the extracted material.

Material eluted from the P-2 column other than the ketocatechol was examined. The only small molecular weight phenol found in Peak II was tyrosine as determined by the UV spectrum and comparison with a tyrosine standard run on high voltage paper electrophoresis. High voltage paper electrophoresis of ^{14}C labeled Peak I and Peak II material from the puparia of animals injected with ^{14}C tyrosine in the third instar showed that this material consists of tyrosine and heterogeneous peptide substances.

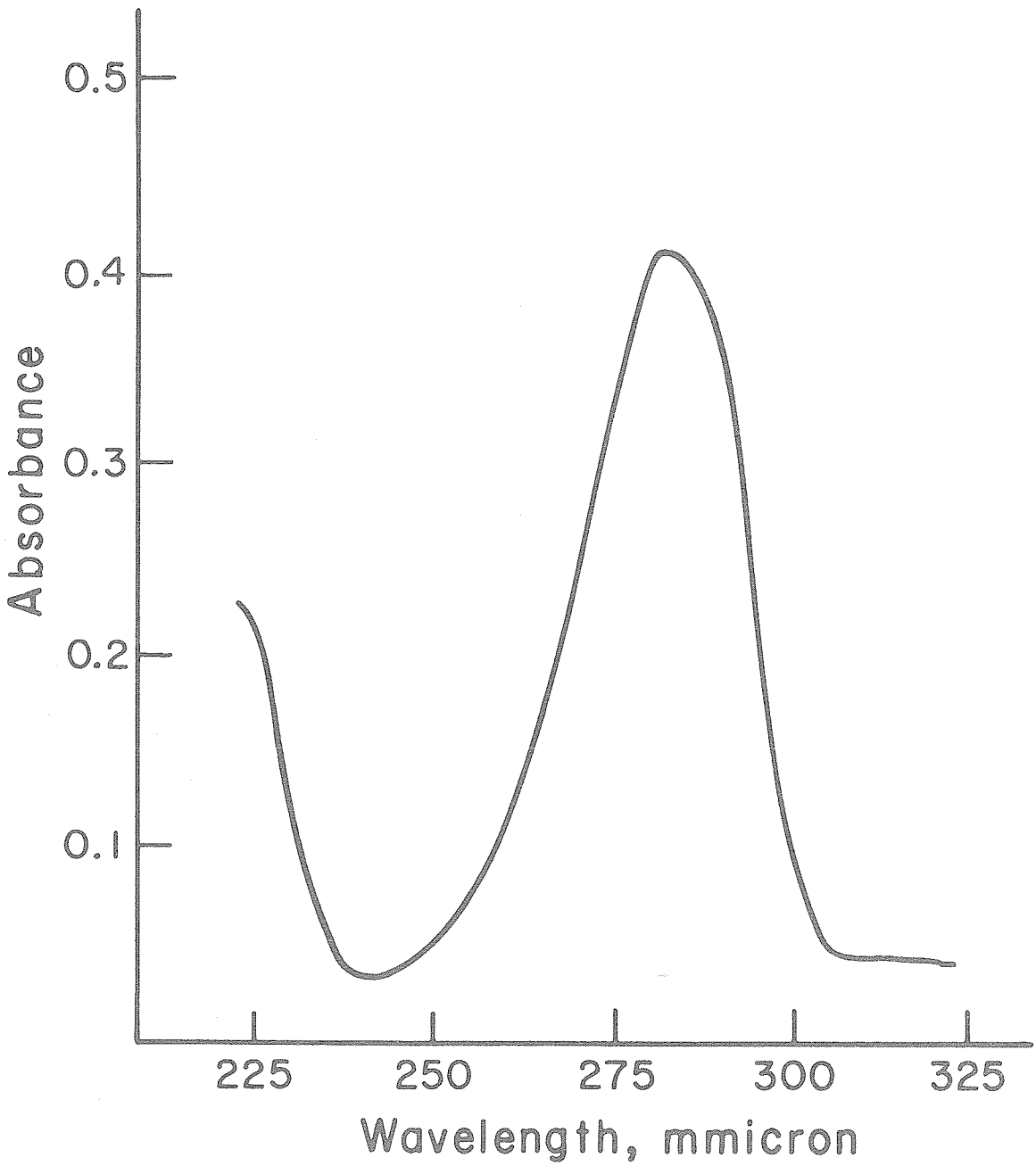
The Peak I and Peak II material (Figure 10) was further hydrolyzed by refluxing in 6N HCl for various times. When the 6N HCl hydrolysate was fractionated by paper electrophoresis and the paper stained with ferric chloride-potassium ferrocyanide reagent, two main spots were observed: one was identified as tyrosine. The other was located about 2 cm from the origin, indicating that the substance was essentially uncharged under the acidic conditions (pH 1.76) of paper electrophoresis. The substance was tentatively identified as a dihydroxyphenol by the almost instantaneous development of the ferric chloride-potassium ferro-cyanide reagent; with phenols, full development takes about 5 minutes. That

the substance was a dihydroxyphenol was further substantiated by experiments described later which showed that from the puparia of animals which had been injected with ^{14}C dopa was extracted this substance strongly labeled. This dihydroxyphenol material had an R_f of 0.4 in the paper chromatography system of n-propanol: water: acetic acid (66:33:1). The UV spectrum of the dihydroxyphenol material which was eluted from paper electrophoresis paper with 0.2N acetic acid is shown in Figure 12. The shoulder at 310 Angstroms characteristic of ketocatechols is not present. The attempt to identify the dihydroxyphenol compound was not pursued further except to note that it evidently is not derived from the puparium substance that is extracted as a ketocatechol, since ketocatechols remain unchanged when subjected to the same extraction conditions as were used to extract the dihydroxyphenol.

An experiment was done to determine if any ketocatechol could be extracted from late third instar larval cuticle. The larval cuticles, approximately 5 mg, were cleaned as much as possible by scraping away tissue with a small spatula. The larval cuticle was extracted in the usual manner by refluxing in 1N HCl. The refluxed material was put over a P-2 column. No peak of UV 280 absorbing material was eluted in the position where ketocatechols are eluted.

The amounts of tyrosine, ketocatechol, and the unknown

Figure 12. UV spectrum of an unidentified dihydroxyphenol in 0.2 N acetic acid. A 1 N HCl puparial hydrolysate from which the ketocatechol material had been separated was further hydrolyzed by refluxing in 6 N HCl for 5 hours. The 6 N HCl hydrolysate was evaporated to dryness, dissolved in 0.2 N acetic acid, and fractionated on high voltage paper electrophoresis. The dihydroxyphenol was eluted from the electrophoresis paper with 0.2 N acetic acid.


0.5
0.4
0.3
0.2
0.1
Absorbance
225
250
275
300
325
Wavelength, mmicron

dihydroxyphenol in the puparium as determined from the extinction coefficients at 280 Angstroms are shown in Table 1. The extinction coefficient for the unknown dihydroxyphenol was taken to be the same as that of dopa, 4300. The extinction coefficient of tyrosine was taken to be 1400; the extinction coefficient of the ketocatechol was taken to be 8200. In Table 1 is also shown the amounts of free tyrosine and free tyrosine-O-phosphate in larvae just before puparium formation and in prepupae just after puparium formation. The values for free tyrosine and tyrosine-O-phosphate were taken from Chen (61).

Radioactive tyrosine and tyrosine derivatives were injected into third instar larvae, and the fate of the label in the puparium determined. In Figure 13 is shown the radioactive profile and the 280 Angstroms absorbance profile of fractions from a P-2 column over which was run the 1N HCl hydrolysis products from puparia of animals injected at 105 hours of the third instar larval stage. In Table 2 is shown the distribution of counts among the three main extractable species - tyrosine, ketocatechol, and dihydroxyphenol - for groups of larvae injected at different times during the third instar with ^{14}C tyrosine. The proportion of counts in the ketocatechol fraction increases the closer to puparium formation that the injection occurs. Comparatively little label is incorporated into pupal case tyrosine when injection is

Table 1

Amounts of Tyrosine and Tyrosine Derivatives in Drosophila*

A.) Amounts extracted from puparia

Compound	nmoles/pupal case
tyrosine	21.5 ± 1.5
ketocatechol	9.6 ± 0.5
dihydroxyphenol	4.0 ± 0.4

B.) Amounts of late third instar larvae and in prepupae after the completion of puparium formation and tanning

	nmoles/animal	
Compound	larvae	prepupae
tyrosine	1.76	0.40
tyrosine-O-phosphate	8.40	0.22

* See text for explanation.

Figure 13. P-2 fractionation of a hydrolysate of puparia from animals injected with $^{14}C(U)$ tyrosine at 110 hours of the third instar. Conditions and methods are the same as described in Figure 10. Fully drawn curve is optical density at 280nm; broken curve is counts per minute.

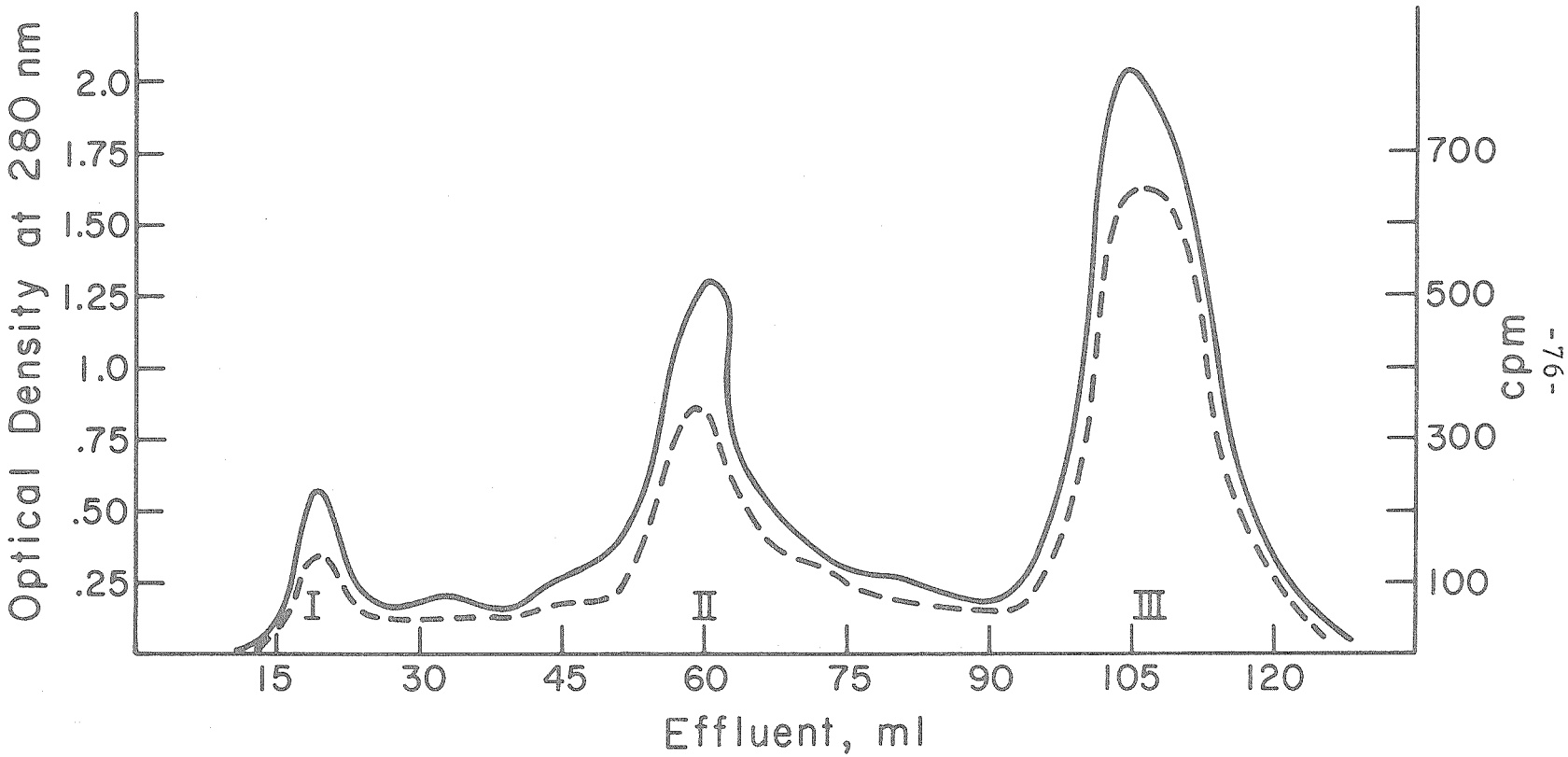

Optical Density at 280 nm
2.0
1.75
1.50
1.25
1.0
.75
.50
.25
I
II
III
15
30
45
60
75
90
105
120
Effluent, ml
700
500
300
100
cpm

Table 2

Distribution of Counts Among the
Pupal Case Extractable Substances

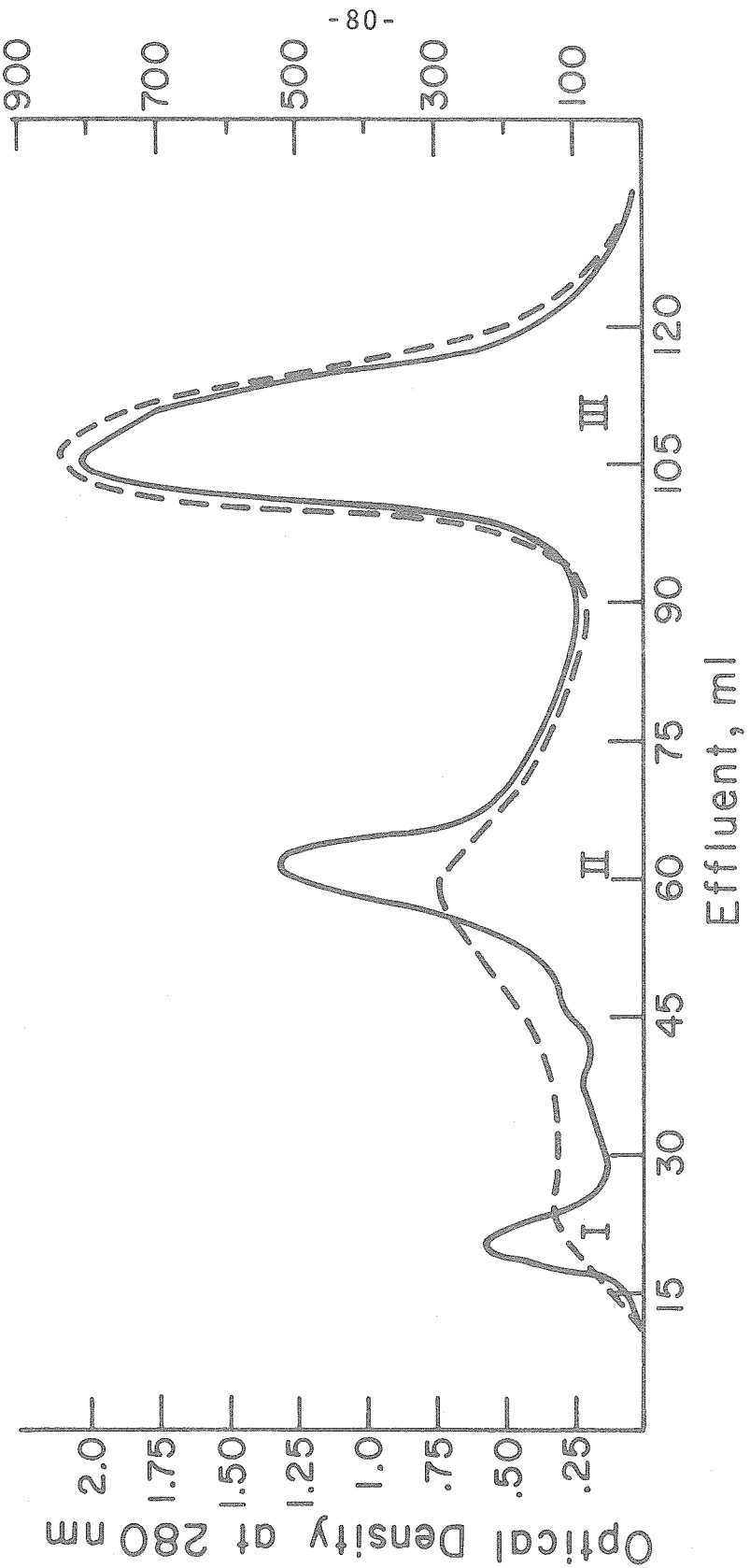
injection time*	COMPOUND		
	ketocatechol	tyrosine	dihydroxyphenol
80 hours	35%	40%	25%
95 hours	44%	33%	23%
110 hours	63%	20%	17%
118 hours	66%	17%	17%

* Larvae were injected at various times during the third instar.

5 hours before puparium formation. The proportion of counts incorporated into the dihydroxyphenol decreases somewhat as the injection time becomes closer to puparium formation. The implications of these data are that the tyrosine which is present in the pupal case was deposited gradually during the larval period and that the large flow of tyrosine into the puparium at puparium formation, which has been described in a previous section, is too a large extent deposited into the puparium in the form of a compound or compounds extractable as a ketocatechol.

Injection and extraction experiments with puparia from animals injected with ^{14}C dopa were also done. In Figure 14 is shown the radioactivity and 280 Angstrom absorbance profile when the 1N HCl hydrolysis products from puparia of animals injected with ^{14}C dopa in the latter part of the third instar was run over a P-2 column. There are no distinct radioactive peaks except for the ketocatechol peak, indicating that the 1N HCl hydrolysis of puparia from dopa ^{14}C injected animals yields heterogeneous labelled peptide products and labelled ketocatechol. The heterogeneity of the peptide products was confirmed by running the hydrolysis products from which the ketocatechol had been separated in high voltage paper electrophoresis; Figure 15a shows the radioactive migration pattern. After hydrolysis of the material shown in Figure 15a by refluxing in 6N HCl for 20 hours, the high

Figure 14. P-2 fractionation of a hydrolysate of puparia from animals injected with ^{14}C dopa at 110 hours of the third instar. Conditions and methods are the same as those described in Figure 10. Fully drawn curve is optical density at 280nm; broken curve is counts per minute.


Optical Density at 280 nm
2.0
1.75
1.50
1.25
1.0
.75
.50
.25
15
30
45
60
75
90
105
120
Effluent, ml
I
II
III
900
700
500
300
100

voltage paper electrophoresis pattern is that shown in Figure 15b. After the 6N HCl hydrolysis much of the label is in a peak very close to the origin; also, there is still some heterogeneous peptide material. The substance in the peak close to the origin had an R_f of 0.4 in the paper chromatography of n-propanol: water: acetic acid (66:33:1); this R_f is the same as that of the unidentified dihydroxyphenol that was extracted from unlabeled puparia and puparia from animals injected with ^{14}C tyrosine.

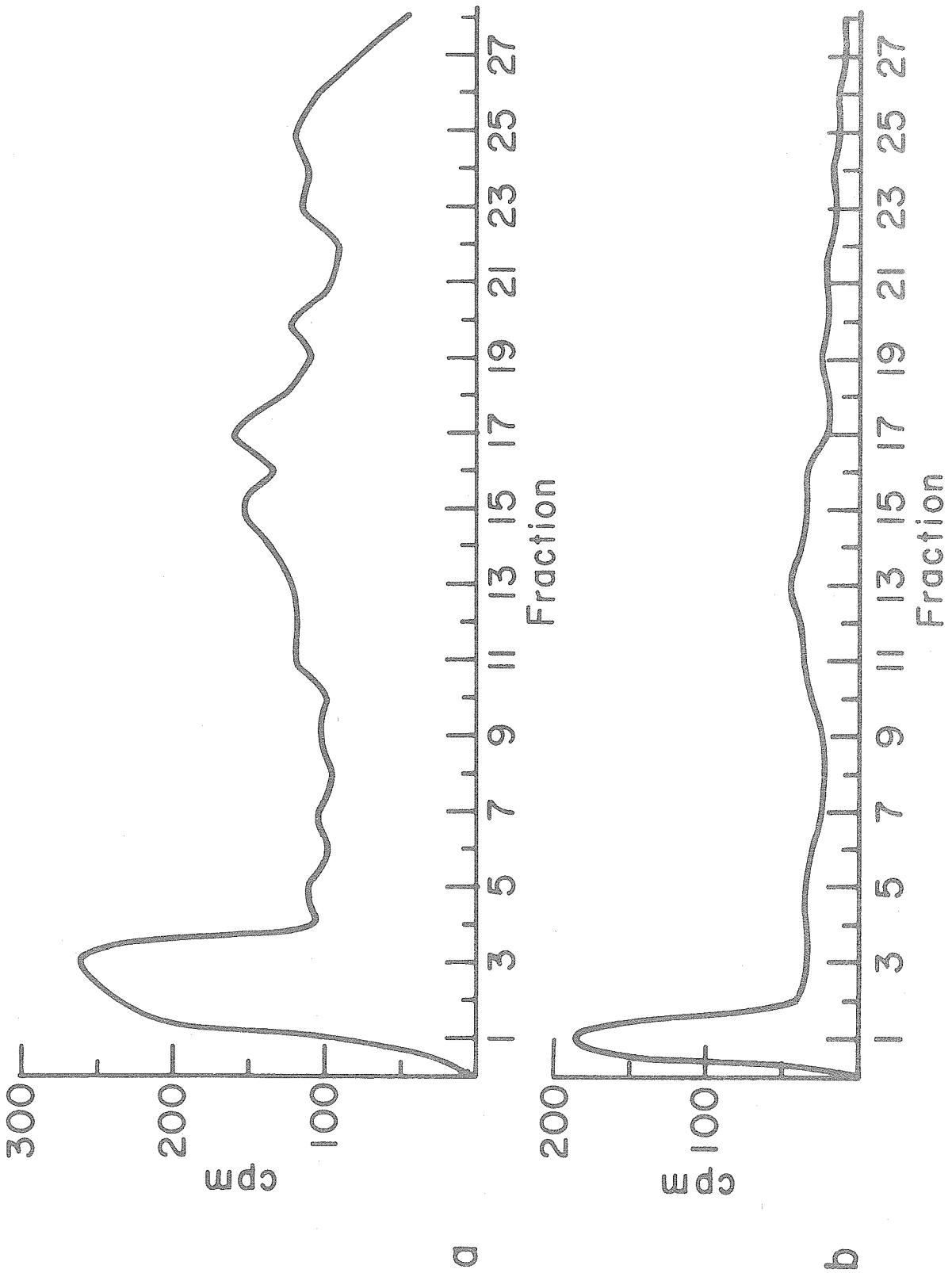
The compound beta-alanine, which has been implicated in the tanning process in Drosophila (61), was injected in the ^{14}C form into late third instar larvae. Pupal cases were collected and extracted by refluxing in 1N HCl. It was found that 90% of the label incorporated into the pupal case was extracted within 30 minutes as beta-alanine. These date indicate that beta-alanine is not incorporated into the puparium by means of a peptide bond.

Enzymatic Degradation of Puparia

As an approach to understanding how the substance extracted with acid as a ketocatechol is incorporated in the puparium, enzymatic degradation of wild type puparia was attempted. The enzymes pronase and chitinase were employed. The general procedure is described in Materials and Methods. In later experiments an extra step was done: before incuba-

Figure 15(a) Radioactivity profile of high voltage paper electrophoresis of 1 N HCl hydrolyzed puparia from ^{14}C dopa injected animals. 110 hour third instar larvae were injected with ^{14}C dopa. Puparia were hydrolyzed and chromatographed on P-2 as described in Figure 10. The ketocatechol peak was separated from the rest of the hydrolyzed material. The hydrolyzed material without the ketocatechol was run on high voltage paper electrophoresis as described in Materials and Methods. After electrophoresis the paper was cut into 1 inch strips; each strip was put into a vial and counted in the scintillation counter.

Figure 15(b) Radioactivity profile of high voltage paper electrophoresis of the same material shown in Figure 15(a) which was hydrolyzed further by refluxing in 6 N HCl for 20 hours. Background of 50 cpm was subtracted.


300
200
100
cpm
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Fraction
a
200
100
cpm
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Fraction
b

tion with enzymes, the puparia were refluxed for 30 minutes in 0.1N NCl in an effort to hydrolyze some of the saccharide bonds in sites hidden from the chitinase. Refluxing in 0.1N HCl for 30 minutes extracts less than 5% of the total ketocatechol.

In preliminary experiments, small quantities - usually about 3 mg - of ^{14}C labelled puparia were used. In these puparia from animals injected with ^{14}C tyrosine 10 hours before puparium formation, ketocatechol extractable substances are preferentially labelled: over 60% of the total counts can be extracted with 1N HCl as ketocatechol. After incubation of the labelled puparia with pronase and chitinase, the insoluble material in the incubation mixture was separated from the soluble material by centrifugation for 30 minutes at 10,000xg in a Servall SS-34 head. The soluble and insoluble fractions of the incubation mixture were separately analyzed to determine the total counts in each and the counts in each extractable as ketocatechol by refluxing in 1N HCl. The results, which are quite reproducible, are shown in Table 3.

The radioactive supernatant from enzyme hydrolysis was fractionated by various methods. In Figure 16 is the radioactive profile of elution from a P-2 column. Over 60% of the label is included; the inclusion limit of P-2 is 2,000 Daltons. The included material appears to be heterogeneous.

Table 3

Solubilization of Puparial Material by

Pronase and Chitinase Enzymolysis

A.) ^{14}C labeled wild type puparia*

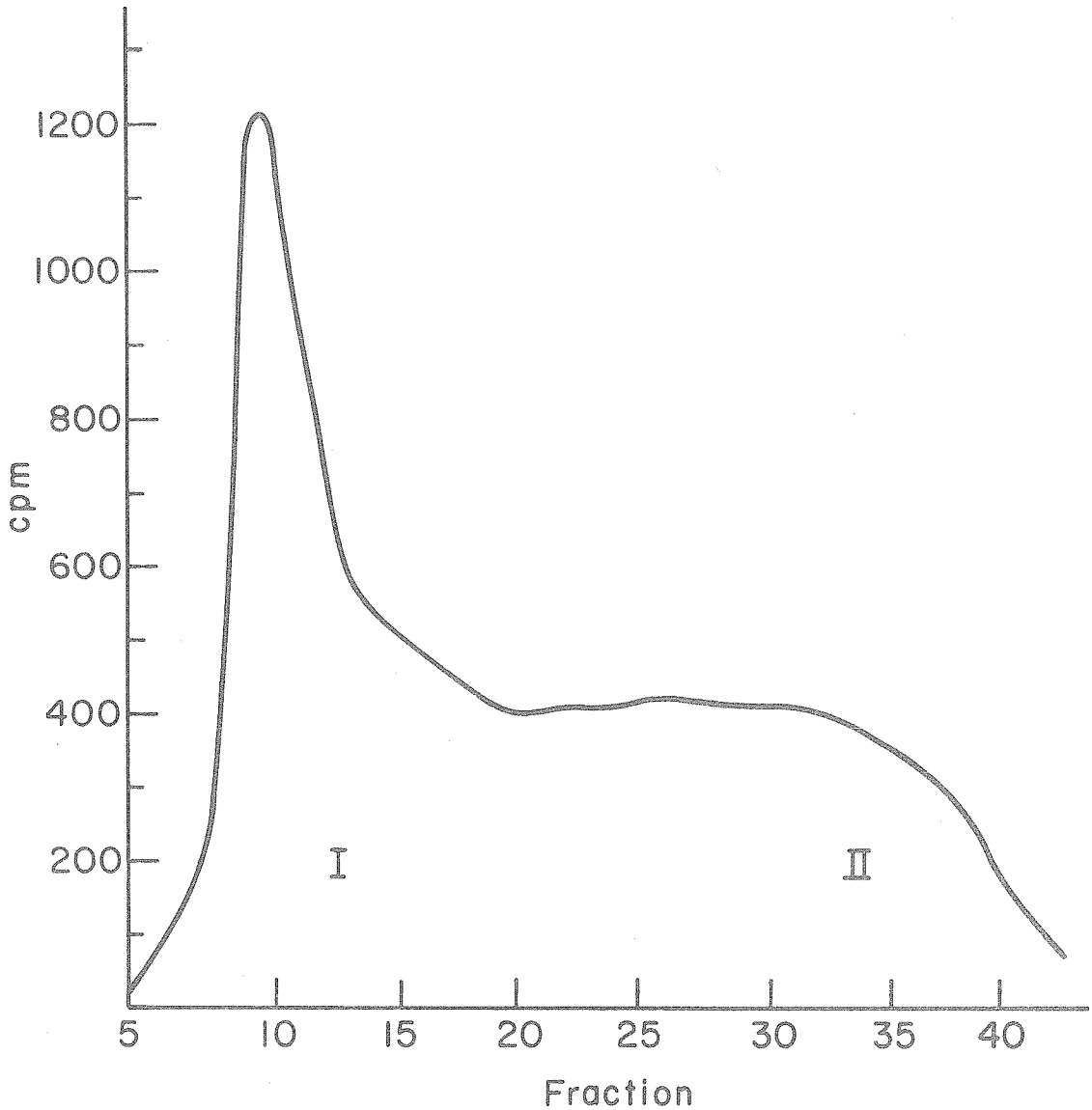
percentage of total counts solubilized	percentage of total 1N HCl ketocatechol extractable counts solubilized
40%	34%

B.) wild type puparia in 100 mg lots*

percentage of total absorbance at 280 Angstroms solubilized	percentage of total 1N HCl extractable ketocatechol solubilized
38%	30%

* No initial step of refluxing puparia for 30 minutes in 0.1N HCl.

Figure 16. Fractionation on BioGel P-2 of the soluble fraction from an enzyme hydrolysis of ^{14}C labelled puparia. Puparia were from animals injected with ^{14}C tyrosine 10 hours before puparium formation. Labelled puparia (3 mg) were incubated with 0.5 mg of chitinase and 0.5 mg of pronase in the manner described in Materials and Methods. The incubation mixture was centrifuged in a Servall SS-34 head at 10,000xg for 30 minutes. The supernatant was redissolved in 1 ml of 0.2 N acetic acid and run over a BioGel P-2 column (2 cm^2 x 25 cm). Elution was performed with .2 N acetic acid.

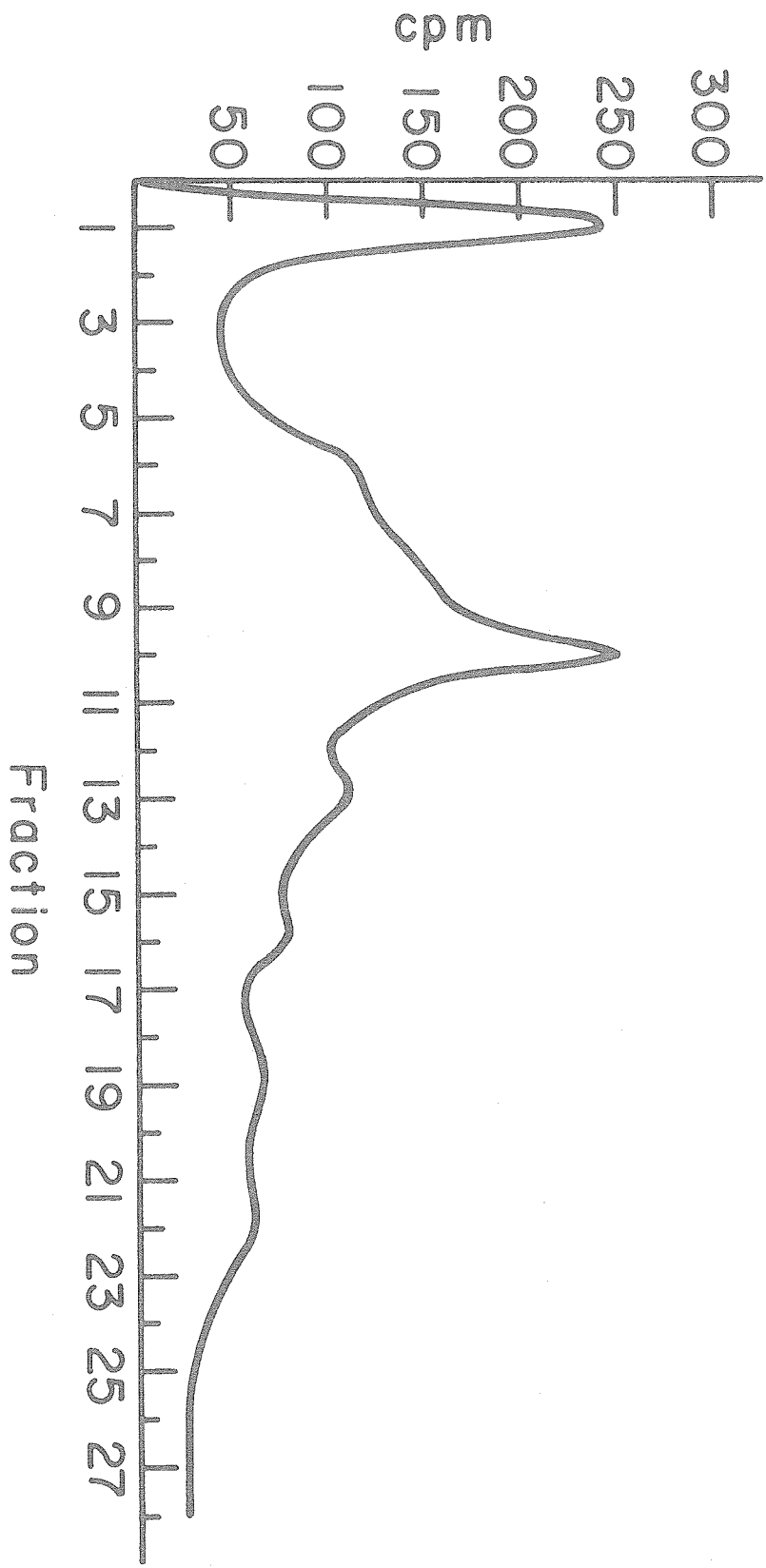

1200
1000
800
600
400
200
cpm
I
II
5
10
15
20
25
30
35
40
Fraction

To determine the distribution of ketocatechol, the effluent was divided at Fraction 13 (Figure 16) into two parts, included and excluded material. The two parts were evaporated and refluxed in 1N HCl to determine the ketocatechol content. In the excluded material approximately 55% of the counts were ketocatechol; in the included material approximately 41% of the counts were ketocatechol. Of the material extractable as ketocatechol in the included volume, most was in the earlier fractions, indicating that the smallest fragment containing this substance is not much less than 2,000 Daltons.

The supernatant from the enzyme degradation of puparia was, also, fractionated by high voltage paper electrophoresis. The radioactive migration pattern is shown in Figure 17. The Fraction 10 peak is composed mostly of tyrosine. The pattern is one of fairly heterogeneous material with possible peaks: especially prominent is the shoulder including Fractions 6, 7, and 8. The enzyme supernatant fractionated by paper electrophoresis in Figure 17 is not completely comparable to the enzyme supernatant fractionated on P-2 in Figure 1b, since the preliminary 0.1N HCl reflux was used in the preparation of the enzyme supernatant fractionated by paper electrophoresis.

Preparation of enzyme hydrolysis products in larger quantities was attempted. Batches of 100 mg of puparia

Figure 17. Radioactivity profile of high voltage paper electrophoresis fractionation of the supernatant from enzyme hydrolysis of ^{14}C labelled puparia. The sample was prepared as described in Figure 16 with the exception of refluxing initially in 0.1 HCl for 30 minutes. Radioactive counting was performed as described in Figure 15.


cpm
50
100
150
200
250
300
1
3
5
7
9
11
13
15
17
19
21
23
25
27
Fraction

were enzymatically hydrolyzed. Ketocatechol content of the supernatant and insoluble fraction of the digest was determined by the standard 1N HCl extraction method. The results are shown in Table 3. UV spectra of the enzyme supernatant showed that the shoulder at 305 Angstroms, characteristic of ketocatechols, was not present. When the soluble fraction of the enzyme digest was lyophilized and redissolved in 4 ml of 0.2N acetic acid in preparation for further fractionation, it was observed that all of the soluble fraction did not go back into solution. Analysis of the material that did not go into solution showed that most of the material extractable as ketocatechol that had been solubilized was present there. Attempts to redissolve the insoluble material by adding as much as 100 ml of 0.2N acetic acid were unsuccessful. Repeated inability to redissolve much of the ketocatechol containing material after lyophilization of the enzyme supernatant prevented attempts to prepare enzyme hydrolysis products in large quantity for study in detail.

Internal Pressure Measurements

Internal pressure in the body cavity of Drosophila was measured at intervals from the third instar to emergence by the technique described in Materials and Methods. The intervlas were between 4 and 8 hours except at three critical periods - puparium formation, wing inflation at 124 hours

from egg-laying, and head eversion at 132 hours, when the intervals were less than 5 minutes. High internal pressure was found only during puparium formation. Soon after the time of the formation of the white prepupa until tanning of the puparium is completed about 3 hours later; the internal pressure remains at a level between 300 and 400 mm Hg. The technique employed does not lend itself to precise pressure versus time measurements, since pressure is measured not continuously but at points and since each animal can be used for only one point. The observation was that the pressure level of 300 to 400 mm Hg is reached within about 15 minutes after the white prepupa is formed and drops to normal within about 30 minutes after sclerotization and tanning are completed.

Increases in internal pressure were expected at certain times during the pupal stage, especially at the time of head eversion at about 12 hours after puparium formation. Head eversion is, as described by Fristrom (75), analogous to the eversion of the indented finger tip of a rubber glove by internal pressure. No change in internal pressure was found during the head eversion period, even though several times the measuring needle was inserted into the pupa during the few seconds during which the head everted. Perhaps, the internal pressure required to evert the head is less than 10 mm

Hg; (the measuring apparatus is not sensitive to pressures less than this). Also, it is possible that the internal pressure is confined in an area of the thorax not penetrated by the measuring needle.

The high internal pressure throughout the whole body during puparium formation is unique during the life cycle of Drosophila and is possibly connected with sclerotization and tanning of the puparium. Based on the observation that Drosophila loses about 20% of its body weight in water during puparium formation, Mitchell et al. (17) suggested that the high internal pressure is a non-specific mechanism whereby the soluble small molecules of the body are forced into the cuticle. The small molecules involved in sclerotization and tanning are incorporated into the cuticle and those not involved are pushed through the cuticle. A preliminary experiment with leucine, a molecule which was found not to be incorporated into the puparium at puparium formation, showed that between 0.5% and 1.0% of the radioactive leucine injected into larvae 5 hours before puparium formation was washed from the cuticle just after puparium formation was completed. Only about 5% of the injected leucine is in the pool of free leucine 5 hours after injection; so, not much more than 1% of the label would be expected to be washed off the cuticle, if the proportion of free leucine lost corresponds to the proportion of water lost at puparium formation. That all of the pool of

free tyrosine and tyrosine-0-phosphate is transported into the puparium is possibly explained by the known accumulation of tyrosine in the epithelial cells during puparium formation (17).

DISCUSSION

Injection of Labelled Tyrosine and Tyrosine Derivatives

The work reported in this thesis was an investigation of the role of tyrosine and tyrosine derivatives in the sclerotization and tanning of Drosophila cuticle. Experiments in which radioactive tyrosine and tyrosine derivatives were injected were designed to determine the pathway from tyrosine to the substances which are actually incorporated into the puparium and to determine how the tyrosine that is incorporated into the puparium at puparium formation is stored in the larva. Experiments, which will be discussed later, that involved degradation of the puparium by various means were designed to determine the structure of the crosslink comprised of tyrosine derivatives.

The fast turnover of dihydroxyphenols in Drosophila made determination of the reaction sequence from tyrosine to the dihydroxyphenol actually incorporated into the puparium uncertain. When ^{14}C tyrosine was injected into third instar larvae, the only small molecule into which label was incorporated before puparium formation was tyrosine-O-phosphate. This observation is contrary to findings for some other insect species: for example, in Calliphora, N-acetyldopamine accumulated before puparium formation and was incorporated intact into the cuticle (34).

In Drosophila, N-acetyldopamine was possibly implicated in sclerotization and tanning. Labelled N-acetyldopamine was observed at a low level for two hours just after puparium formation after injection of radioactive tyrosine into late third instar larvae. This short appearance of N-acetyldopamine suggests that the rate of incorporation slows down as the cross-linking sites in the puparium become filled, allowing a small accumulation.

Injection experiments showed that N-acetyldopamine is synthesized from injected radioactive dopa and dopamine throughout the third instar; the turnover was rapid with all label within a few hours being incorporated into insoluble material. This synthesis throughout the third larval instar possibly indicates some metabolic function in Drosophila of N-acetyldopamine in addition to the postulated role of sclerotizing agent. As described in the Results section there is some difficulty in the interpretation of the radioactive dopa, dopamine, and N-acetyldopamine injection results because of the very low natural level of these substances in Drosophila larvae. The results of Mitchell and Lunan (49), which show a large peak of dopa decarboxylase activity near puparium formation of Drosophila, are strong evidence that N-acetyldopamine or some other dopamine derivatives are involved in sclerotization. Hodgetts and Konopka (70) measured dopamine N-acetylase activity and found activity in mature third instar larvae and pharate adults;

However, they show no data to indicate the change in activity with time through the larval and pupal stages.

Radioactive tyrosine injection indicated that the tyrosine that is incorporated into the puparium at puparium formation is derived mainly from the pool of free tyrosine and tyrosine-O-phosphate. Similar experiments done by Fraenkel *et al.* (51) with newly emerged *Sarcophaga* adults suggested that there is some storage form for tyrosine other than the pool of free tyrosine and tyrosine-O-phosphate, which is found in *Sarcophaga*. The dilution of radioactive free tyrosine by non-radioactive tyrosine before sclerotization and tanning was more than could be accounted for by the hydrolysis of tyrosine-O-phosphate. Fraenkel *et al.* postulated that the tyrosine that accumulated before sclerotization and tanning was derived from the turnover of proteins. Another form exists in *Sarcophaga* larvae for the storage of tyrosine before puparium formation. Bodnaryk and Levenbook (71) found large quantities of a beta-alanine-tyrosine dipeptide, which they called sarcophagine. At puparium formation, the tyrosine and beta-alanine which compose sarcophagine are incorporated into the puparium.

The role of tyrosine-O-phosphate in *Drosophila* has never been very clear. The thesis, more evidence for which will be discussed later, that the pool of free tyrosine and tyrosine-O-phosphate furnish all the tyrosine incorporated into the puparium at puparium formation points up the importance of

tyrosine-O-phosphate as a source for the tyrosine used at puparium formation. The high solubility of tyrosine--O-phosphate allows the organism to hold a tyrosine supply sufficient for puparium formation in the form of a soluble small molecule. As a rough estimate, the free tyrosine concentration in third instar larvae is 70% of the saturation point for tyrosine (see Table 1). The amount of tyrosine necessary for puparium formation is much more than is soluble as free tyrosine.

As mentioned previously, in other insects general proteins or tyrosine rich proteins and peptides have been proposed as tyrosine storage forms. Certain advantages are derived from storing tyrosine as a small soluble molecule rather than in a large polymerized form. One advantage is the ability to pass through cell membranes. At puparium formation in Drosophila all the free tyrosine and tyrosine-O-phosphate is transported rapidly to the epithelial cells where it is present in high concentration during puparium formation. Autoradiographic studies of the mobilization of these molecules were done by Mitchell et al. (17) . If tyrosine were stored in protein form, hydrolysis of the protein to allow passage through cell membranes to the epithelial cells would be necessary unless the protein were transported in some specialized form or unless the protein itself were stored in the epithelial cells.

If the protein were hydrolyzed to release free tyrosine for transportation to the epithelial cells, at least in the case of Drosophila, the free tyrosine would still probably have to be converted to some more soluble form such as tyrosine-O-phosphate in order that the high concentration of phenol material could be maintained in the epithelial cells during puparium formation. Probably, the high concentration of phenol material in the epithelial cells is necessary so that puparium formation may be accomplished comparatively rapidly. Mitchell et al. (17) stated that the limiting step in providing material for accumulation in the puparium appears not to be in epithelial cell metabolism but in availability of tyrosine from the hemolymph. A function such as tyrosine-O-phosphate might serve in Drosophila might not be needed in insects in which sclerotization proceeds much more slowly: for example, in the locust, sclerotization of new cuticle takes several days to accomplish.

Size of the insect could have some bearing on the storage of tyrosine. Insects larger than Drosophila with a lower surface to volume ratio might be able to store enough tyrosine in the free state for use in sclerotization, although a more soluble derivative like tyrosine-O-phosphate would be necessary if phenol material were concentrated in the epithelial cells during sclerotization.

Tyrosine-O-phosphate is not an inert but a dynamic storage form. Radioactive tyrosine-O-phosphate injection experiments showed a rapid turnover in the form of a tyrosine-tyrosine-O-phosphate exchange throughout the third larval instar. What the energy expended in the breakage and resynthesis of the high energy phosphate bond of tyrosine-O-phosphate accomplishes in the larval metabolism is not known.

Acidic Degradation of Puparia

Acidic degradation of puparia yielded large amounts of ketocatechol, the extraction of which was first described by Andersen and his co-workers (37, 38, 39). The puparium can be assumed to contain some catechol derivatives which on treatment with acid give rise to the ketocatechol structure; these catechols must be firmly bound since they cannot be extracted except under fairly strong conditions. From Drosophila pupal cases one kind of ketocatechol was extracted, 2-hydroxy-3',4'-dihydroxyacetophenone. Andersen reported the extraction of several kinds of ketocatechols; but, when the extraction was done under nitrogen only 2-hydroxy-3',4'-dihydroxyacetophenone was extracted. As pointed out by Andersen (37) and confirmed by these experiments, the catechols which give rise to ketocatechols are not present in cuticle as ketocatechols, since enzymatic digests do not give the charac-

teristic ketocatechol UV-spectrum.

It appears that the tyrosine that flows into the puparium at puparium formation is incorporated almost entirely as material extractable as ketocatechol. This is indicated by three observations: (1) The closer to puparium formation that ^{14}C tyrosine is injected the higher the proportion of counts extracted from the puparium as ketocatechol (see Table 2).(2) No ketocatechol can be extracted from larval cuticle; however, this does not rule out the possibility that phenol or diphenol derivatives already present in the cuticle might be converted to substances extractable as ketocatechol. (3) Previous experiments in which the fate of ^{14}C tyrosine was followed showed that only the pool of free tyrosine and tyrosine-O-phosphate was incorporated into the puparium. Table 1 shows that the quantity lost from the pool of free tyrosine and tyrosine-O-phosphate is very close to the quantity of ketocatechol extracted from the puparium.

Possible participation of quinone crosslinks in puparium formation in terms of quantity appears to be small. It was determined that over 90% of the tyrosine and tyrosine derivatives in the puparium were extracted with 1N HCl. Most of the extracted material could be accounted for in the ketocatechol, tyrosine, and unidentified dihydroxyphenol fractions. Lipke and Geoghegan (27) in their analysis of the degradation prod-

ucts of the cockroach cuticle also came to the conclusion that the quinone crosslinks were not prominent in that cuticle.

Quinone crosslinks would not be hydrolyzed under any of the conditions described in the experiments of this thesis. After complete acid hydrolysis of the puparium, material containing quinone crosslinks would probably be present as fragments of different sizes depending upon the number of quinone crosslinks. It can be seen in Figure 15, which shows the radioactive profile of material - from which the ketocatechol fraction had been removed - from puparia of animals injected with ^{14}C dopa, that the possible quinone containing material in the fractions past the peak of unidentified dihydroxyphenol near the origin is small compared to nonquinone substances. Andersen and Barrett (38) postulated that the existence of a ketocatechol generating crosslink does not rule out the possibility of quinone participation in crosslinking, especially in darker cuticles. Almost certainly tan Drosophila puparia do contain quinone crosslinks, since the pigment of various insects including the puparial pigment of Sarcophaga (57) has been shown to be melanin, which contains quinone crosslinks. In Drosophila puparia, the amount of tyrosine converted to quinones in melanin must be relatively small.

Tyrosine and an unidentified dihydroxyphenol species are two other prominent tyrosine derivatives which can be extracted with acid. The tyrosine is derived from the hydrolysis of

cuticle protein. Injection experiments showed that radioactive tyrosine is incorporated into the cuticle as tyrosine throughout the third larval instar. The cuticle is growing during the third instar from a thickness of about 3 microns at the beginning to a thickness of about 25 microns just before puparium formation (17). The dihydroxyphenol was extracted in an amount about one-half that of the ketocatechol. That the dihydroxyphenol is not derived from the same material from which ketocatechols are was shown by the fact that ketocatechols remain unchanged under the more severe hydrolysis conditions used to extract the dihydroxyphenol. Lipke and Geoghegan (27) extracted, by similar hydrolysis conditions from cockroach cuticle, a dihydroxyphenol which was tentatively identified as dihydroxybenzoic acid. Injection experiments (see Table 2) indicate that the dihydroxyphenol is not derived from tyrosine incorporated at puparium formation. It was also found that this dihydroxyphenol does exist in larval cuticle.

It will be recalled that in most of the experiments described in this thesis the tyrosine metabolism of wild-type has been compared with that of ebony, which has a white puparium. No significant differences were found. It is known that ebony does not incorporate nearly as much beta-alanine into its puparium as does wild-type (61). Beta-alanine was hydrolyzed from puparia by acidic conditions much milder than

are required for complete hydrolysis of peptide bonds. Bodnaryk and Levenbook (71) reported that beta-alanine is present in Sarcophaga puparia mainly as the N-terminal amino acid of cuticle proteins. Their method consisted of incubating ground puparia with DNP and determining the amino acids linked with DNP. DNP attaches to any free amine group, not necessarily just the N-terminal group of proteins. That beta-alanine was hydrolyzed from the puparia by the relatively mild conditions reported here suggests that beta-alanine is bound to the cuticle through, perhaps, an ester linkage with the amine group free.

Enzyme Degradation

Enzyme degradation of puparia was done to see if the sclerotizing and tanning crosslinks could be solubilized and isolated for analysis. The enzyme degradation solubilized somewhat less than one-half of the material containing substances extractable as ketocatechol. Incomplete digestion by enzymolysis is characteristic of sclerotized insect cuticle (37, 72). Lipke (40), working with Sarcophaga puparia, tried various proteases coupled with chitinase and chitobiase. He succeeded in breaking 5% of the bonds that are normally cleaved by these enzymes. Much of the soluble material was in the form of glyco-peptides in the 1000 to 3000 molecular weight range.

Lipke attributed the incomplete digestion to hydrogen bonding in the cuticle which masks most of the peptide and saccharide bonds from enzymolysis.

That the crosslink extractable as a ketocatechol can be solubilized by enzymolysis offers hope that it may eventually be isolated. The heterogeneity in size and charge of the solubilized fragments containing this crosslink was demonstrated by fractionation on columns of BioGel P-2 and P-5, Sephadex G-15, and Whatman Cellulose phosphate and by fractionation by high voltage paper electrophoresis. The smallest fragments containing this crosslink were barely included on P-2, which has an exclusion limit of 2000 Daltons. Whether these fragments of somewhat less than 2000 Daltons carry the actual crosslink and contain no more bonds that can be cleaved by the enzymes employed or whether these fragments still contain bonds which can be cleaved but are hidden from the enzymes is not known.

Isolation of the crosslink would be expedited if a method of enzymolysis could be found which gives more complete hydrolysis than the method employed in the experiments described here. Judging from the previously cited results of Lipke (40), it is likely that the enzymolysis observed here is near the maximum obtainable with enzymes alone. Prior light acid hydrolysis, which did not disrupt the crosslinks, increased

the amount of crosslinks solubilized by about 15% over the amount solubilized by enzymolysis alone. Preliminary fractionations of the soluble fraction of the dilute acid and enzyme treated puparia showed heterogeneity of the fragments containing the crosslink and a generally lower molecular weight of the fragments in comparison with the products of enzymolysis alone.

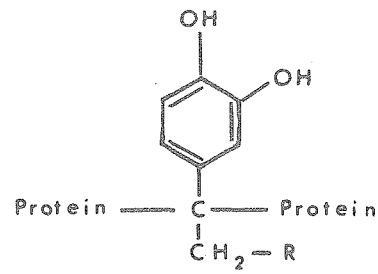
The heterogeneity of the fragments produced by enzymolysis might to some extent reflect the mixed nature of the crosslinks which they contain. Differences among crosslinks might be accounted for by differing numbers of catechols involved in each crosslink as well as differences in quantity and type of other unknown crosslink constituents. Some work of Andersen (39), which will be discussed later, suggests how a variety of different crosslinks containing catechols extractable as ketocatechol could come about. If the crosslink is heterogeneous, isolation of sufficient amounts to analyze will be difficult. Partial acid hydrolysis might yield homogeneous parts of crosslinks which could be isolated.

Speculations on the Nature of the Crosslink

Working from the foundation established by Andersen and his co-workers (37, 38, 39), it has been determined that the quinone crosslink which has been considered to be responsible

for sclerotizing insect cuticle does not play a major role in the sclerotization of the Drosophila puparium; and the question of the nature of the crosslink remains.

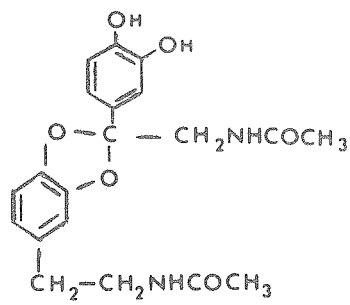
Because the catechol extractable as a ketocatechol is incorporated into the puparium at puparium formation in large amounts, it is likely that the sclerotizing crosslink is made by this catechol. For a ketocatechol to be extracted, there must have occurred at some time an oxidation of the side-chain beta-carbon. Andersen (38) pointed out that since this carbon atom is in the oxidized state when extraction is performed under anaerobic conditions, it can be assumed that the oxidation takes place in the animal; Andersen (38) proposed that the cuticle becomes sclerotized by a reaction involving oxidation of the beta-position of the side-chain of catechols which results in the structure illustrated below:



if N-acetyldopamine is the sclerotizing agent, R would be $-NHCOCH_3$.

Evidence was presented that an enzyme able to catalyze such a crosslink is present in locust cuticle (39). In vitro experiments showed that locust cuticle, free of any adhering

cells, when incubated with N-acetyldopamine labelled with tritium in the side-chain, was able to remove tritium from the beta-carbon. After removal of the tritium the N-acetyldopamine either became incorporated into the cuticle, from which it could be extracted as a ketocatechol, or reacted with other N-acetyldopamine molecules to form soluble compounds. One of these compounds was tentatively identified as illustrated below:

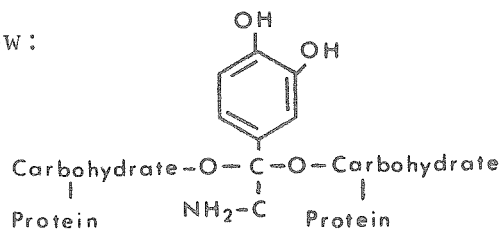


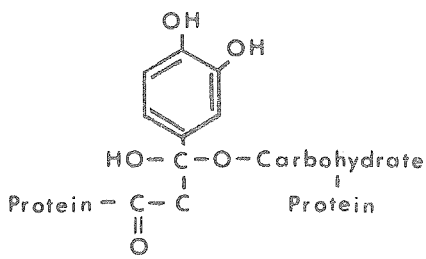
Such a structure suggests why the actual crosslink may be of a mixed nature since linkage of catechols through the reactive beta-carbon to hydroxyl groups of catechols already incorporated appears possible.

Andersen (39) suggested that the activated beta-carbon crosslinks protein by reacting with N-terminal amino groups, free lysine amino groups, and tyrosine hydroxyl groups. The evidence for this was that many of these groups are not free to react with blocking agents after sclerotization. The possibility that these groups were not accessible to the blocking agents was not eliminated.

Other types of crosslinks that would give the ketocatechol structure are possible. A crosslink in which a catechol is

bonded to a protein-attached carbohydrate through an ether linkage at the beta-carbon to form a ketal would be hydrolyzed to give a ketocatechol. Also giving a ketocatechol is a crosslink in which the beta-carbon of dopamine is joined to a protein-attached carbohydrate to form a hemiketal and the alpha-carbon amino group is joined by an acyl linkage to an aspartic acid of another protein. The two proposed links are illustrated below:





Lipke and Geoghegan (27) reported that carbohydrates are involved in the sclerotization of the cockroach cuticle. Glucose, pentose, and mannose were recovered from hardened puparia when it was hydrolyzed by acid; the amount of hexoses incorporated into the cuticle increased sharply during sclerotization.

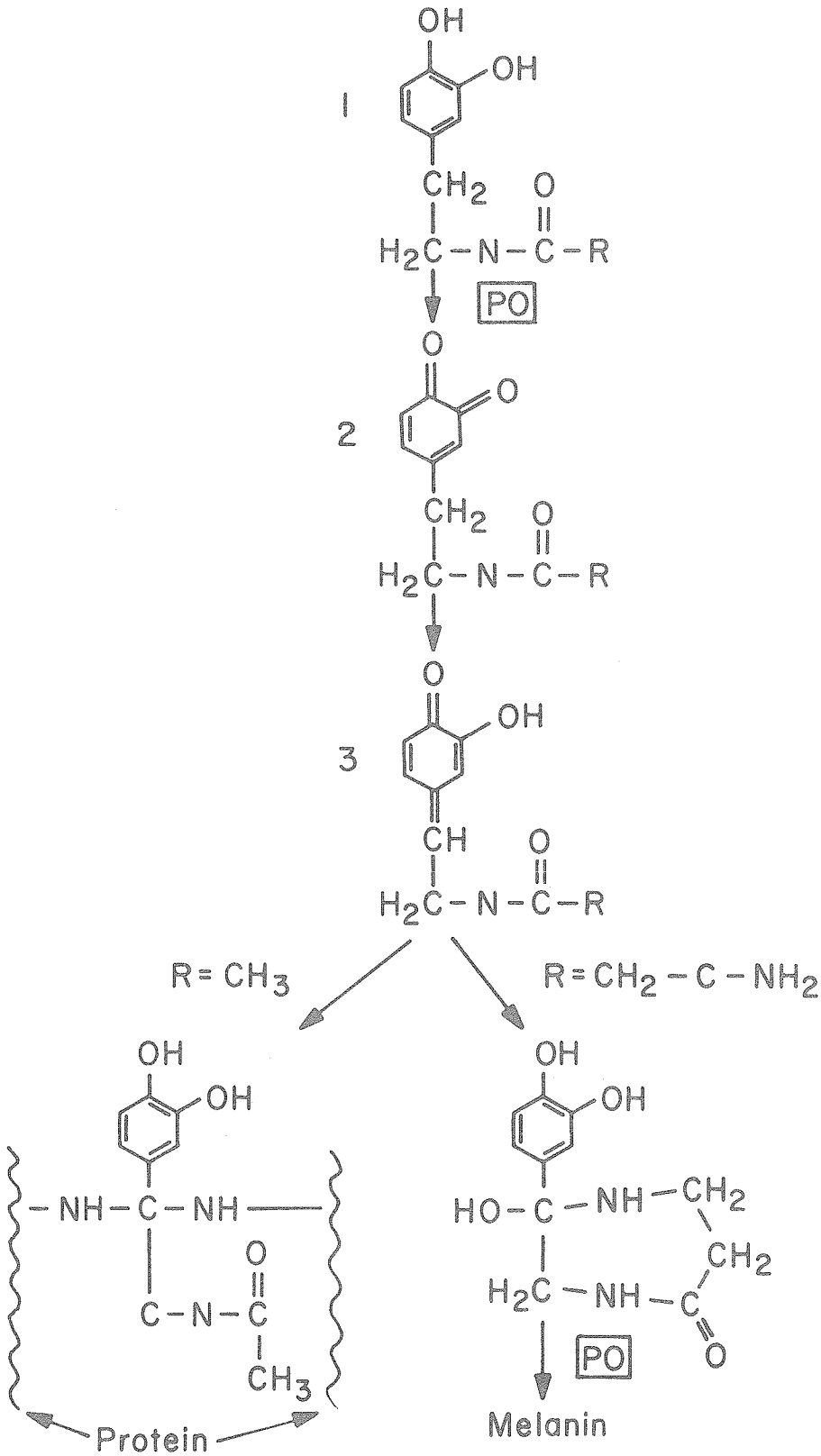
The sort of enzyme that catalyzes the reaction that produces the crosslink extractable as ketocatechol is not known. The enzyme dopamine beta-hydroxylase which is involved in the synthesis of adrenalin is a well characterized enzyme that may be analogous to an enzyme involved in the synthesis of the cuticle crosslink. The structural characteristics necessary for substrate activity for this enzyme are an aro-

matic ring with a side-chain of two or three carbon atoms terminating in an amino group. Cofactor requirements for the adrenal gland dopamine beta-hydroxylase include ATP and ascorbic acid (74). Most likely such cofactors are not available in the cuticle.

Little is known about the enzymes of the insect cuticle. Proteins are not normally extracted from the cuticle except by conditions which would destroy enzyme activity. Andersen's (39) *in vitro* cell free cuticle synthesis of the ketocatechol extractable crosslink showed that all the enzymes necessary for the synthesis with N-acetyldopamine as substrate are located in the cuticle. Most insect cuticles contain phenoloxidase (33, 54, 55). The insect hemolymphal phenoloxidase has been extensively studied; whether the cuticle phenoloxidase and the hemolymphal phenoloxidase are the same is not known. Undoubtedly, cuticle phenoloxidase is involved in the synthesis of the pigment, melanin (56, 57).

In Figure 18 is a scheme which shows how the reactions involved in sclerotization and tanning might be catalyzed by phenoloxidase. The strong electron withdrawing character of the hydroquinone depicted as structure 3 would make the beta-carbon very reactive toward nucleophilic groups such as amino and hydroxyl groups. In the reaction sequence, R is either part of an acetyl group or part of beta-alanine. If R is part of an acetyl group, the beta-carbon reacts with free amine

Figure 18. A scheme that describes possible reactions catalyzed by phenoloxidase in the sclerotization and pigmentation of insect cuticle.


1
2
3
PO
R = CH3
R = CH2 – C – NH2
Protein
Melanin

or hydroxyl groups on cuticle protein, crosslinking it. If R is part of beta-alanine, the free amine group of beta-alanine reacts with the beta-carbon, causing it to be deactivated. Phenoloxidase oxidizes the deactivated catechol, which polymerizes with other quinones to form melanin. The Drosophila puparium is not tanned unless beta-alanine is incorporated (61).

The question arises whether tyrosine is the only molecule constituting sclerotizing crosslinks. The belief in the idea of the quinone crosslink has tended to limit investigation of sclerotization to the aspect of tyrosine involvement. According to Chen (42), of the free amino acids of insects, only tyrosine and proline increase near the time of sclerotization. Possible involvement of proline in sclerotization should be investigated. Experiments not reported in this thesis showed that free leucine and alanine are not incorporated into the Drosophila puparium at puparium formation, suggesting that there is not a general incorporation of amino acids at this time. Lipke (41) showed that glycosylation reactions involving hexoses incorporated into the cuticle during sclerotization contribute to sclerotization. The interaction of the proteins and carbohydrates of the insect cuticle is not understood.

The problem of how the insect cuticle is sclerotized is not solved. The dogma of sclerotization by the quinone crosslink, which, in retrospect, has been believed with little supporting evidence, may be found to be generally invalid.

The data of this thesis showed that the number of possible quinone crosslinks in the puparium must be small compared to the number of crosslinks extractable as ketocatechol. Of the tyrosine incorporated into the Drosophila puparium at puparium formation, almost all can be extracted as ketocatechol. Andersen, who discovered the ketocatechol extractable crosslink, has extracted ketocatechols from the sclerotized cuticles of different species of insects (38). The next step in the understanding of sclerotization must be the isolation and analysis of the sclerotizing crosslink.

REFERENCES

1. Wigglesworth, V.B., in The Principles of Insect Physiology, pp. 29-60, Chapman and Hall, London(1965).
2. Richards, A.G., in Insect Physiology (K.D. Roeder, ed.) pp. 1-23, John Wiley and Sons, N.Y. (1953).
3. Wigglesworth, V.B., in The Life of Insects, pp. 1-40, World Publishing Co., N.Y. (1964).
4. Weis-Fogh, T., in Insect Ultrastructure (A.C. Neville, ed.) pp. 163-185, Blackwell Scientific Publications, Oxford (1970).
5. Neville, A.C., in Insect Ultrastructure (A.C. Neville, ed.) Blackwell Scientific Publications, Oxford (1970).
6. Locke, M., J. Morph., 118: 461-494 (1966).
7. Andersen, S.O., Acta Physiol. Scand., 66:1 (1966).
8. Neville, A.C. and Luke, B.M., Tissue and Cell, 1: 689-707 (1969).
9. Baker, G., Peppar, J.H., and Johnson, L.H., J. Ins. Physiol., 5:47-60 (1960).
10. Bursell, E. and Clements, A., J. Ins. Physiol., 13: 1671-8 (1967).
11. Bowers, W.S. and Thompson, M.J., J. Ins. Physiol., 11: 1003-11 (1965).
12. Dennell, R. and Malek, S.R.A., Proc. R. Soc., B 143: 239-257 (1955).

13. Dennell, R. and Malek, S.R.A., Proc. R. Soc., B 144: 545-556 (1956).

14. Fox, F.R. and Mills, R.R., Comp. Biochem. Physiol., 29: 1187-1195 (1969).

15. Jeuniaux, C., Bull. Soc. Chim. Biol., 47:2267-2278 (1965).

16. Condoulis, W.V., and Locke, M., J. Ins. Physiol., 12: 311-323 (1966).

17. Mitchell, H.K., Weber-Tracy, U.M., and Schaar, G., J. Exp. Zool, 176:429-443(1971).

18. Stevenson, J.R. and Adomako, T.Y., J. Ins. Physiol., 13: 1803-1813 (1967).

19. Coles, G.S., J. Ins. Physiol., 12:679-691 (1966).

20. Stern, C., Rev. Suisse Zool., 73:339-357 (1966).

21. Sinex, R.M., in Treatise on Collagen (Bernard S. Gould, ed.), pp. 410-467 (1968).

22. Partridge, S.M., Elsden, M.H., and Thomas, J., Nature, 197:1297-1298 (1963).

23. Perkins, H.R., Bacteriol. Rev., 27:18-55 (1963).

24. Hackman, R.H., J. Ins. Physiol., 17:1065-1071 (1971).

25. Degens, T., Carey, F.G., and Spencer, D.W., Nature, 216: 601-603 (1967).

26. Rudall, K.M., Adv. Ins. Physiol., 1:257-311(1963).

27. Lipke, H., and Geoghegan, T., J. Ins. Physiol., 17:415-425(1971).

28. Pryor, M.G.M., Proc. Roy. Soc., B 128: 378-384 (1940).

29. Pryor, M.G.M., Proc. Roy. Soc., B 128:384-393 (1940).

30. Brunet, P.C.J. and Kent, P.W., Proc. Roy,Soc., B 144: 259-270 (1955).

31. Mason,H.S., in Advances in Enzymology (F.F. Nord,ed.) Vol.16, p.105, Interscience Publishers, N.Y. (1955).

32. Brown, H., Nature, 165: 275 (1950).

33. Pryor, M.G.M., in Comparative Biochemistry , (M. Florkin and H.S. Mason, eds.) Vol. 3, p. 471, Academic Press, N.Y. (1964).

34. Karlson, P. and Sekeris, C.E., Biochem. Biophys. Acta, 63: 489 (1962).

35. Karlson, P., Sekeris, C.E. and Sekeris, K.E., Hoppe-Seyler's Z. Physiol. Chem., 327: 86 (1962).

36. Karlson, P. and Sekeris, C.E., Nature, 195: 183 (1962).

37. Andersen, S.O., J. Insect Physiol., 16:1951 (1970).

38. Andersen, S.O., and Barrett, F.M., J. Insect Physiol., 17: 69 (1971).

39. Andersen, S.O., J. Insect Physiol., 18: 527 (1972).

40. Lipke, H., Insect Biochem., 1: 189 (1971).

41. Lipke, H., Grainger, M., and Siakotos, A., J. Biol. Chem., 240: 594 (1965).

42. Chen, P.S., in Advances in Insect Physiology (J.W.L. Beament, J.E. Trherne and V.B. Wigglesworth, eds.) Vol. 3, p. 53, Academic Press, N.Y. (1966).

43. Cottrell, C.B., J. Expt. Biol., 39: 431 (1962).

44. Dennel, R., Proc. Roy. Soc., B 134: 163 (1947).

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

46. Sekeris, C.E. and Herrlich, P., Hoppe-Seyl. Z., 344: 267 (1966).

47. Mills, R.R., Lake, C.R. and Alworth, W.L., J. Insect Physiol., 13: 1539 (1967).

48. Mitchell, H.K., Chen, P.S. and Hadorn, E., Experientia, 16: 1 (1960).

49. Mitchell, H.K. and Lunan, K.D., Arch. Biochem. Biophys., 106: 219 (1964).

50. Lunan, K.D. and H.K. Mitchell, Arch. Biochem. Biophys., 132: 450 (1969).

51. Seligman, M., Friedman, S., and Fraenkel , G., J. Insect Physiol., 15: 1085 (1969).

52. Geltosky, J., personal communication.

53. Yamazaki, H.I., J. Insect Physiol., 15: 1803 (1967).

54. Blumenthal, A., Thesis, California Institute of Technology (1971).

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

56. Hackman, R.H., Nature, 216: 63 (1967).

57. Fogal, W. and Fraenkel, G., J. Insect Physiol., 15: 1235

(1969).

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

59. Jacobs, M.E., Genetics, 53: 777 (1966).

60. Fukushi, Y., Jap. J. Genetics, 42: 11 (1967).

61. Hodgetts, R.B., J. Insect Physiol., 18: 937 (1972).

62. Tanzer, M., Science, 180: 561 (1973).

63. Mitchell, H.K. and Mitchell, A., Drosophila Information Service, 39: 134 (1964).

64. Boyd, J.B., Thesis, California Institute of Technology (1966).

65. Mitchell, H.K., personal communication.

66. Fodor, Y. and Kovacs, M., J. Am. Chem. Soc., 71: 1045 (1949).

67. Dreyer, W. and Bynum J., in Methods in Enzymology, (C.W. Hirs, ed.) Vol. 21, Academic Press, N.Y. (1967).

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

69. Mitchell, H.K., personal communication.

70. Hodgetts, R.B. and Konopka, R.J., J. Insect Physiol., 19: 1211 (1973).

71. Bodnaryk, J. and Levenbook, A., Comp. Biochem. Physiol., 30: 909 (1969).

72. Hackman, R.H., Aust. J. Biol. Sci., 13: 568 (1960).

73. Lake, C.R., Mills. R.R. and Brunet, P.C.J., Biochem. Biophys. Acta, 215: 226 (1970).

74. Molinoff, P.B. and Axelrod, J., in Annual Review of Biochemistry , Vol. 40, p. 451 (1971).

75. Fristrom, J.W., Genetics, 52: 297 (1965).