

TYROSINE AND PHENYLALANINE CONCENTRATIONS IN HAEMOLYMPH
AND TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA
AMERICANA (L.) DURING METAMORPHOSIS

by

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INTRODUCTION

The aromatic amino acid tyrosine plays a many faceted role in the physiology of insects. Aspects of its metabolism have been reviewed several times in the last decade (Brunet, 1963, 1965, 1967; Cottrell, 1964; Hackman, 1964).

The free amino acids in insect haemolymph are thought to function in osmoregulation, buffering, and possibly in protection from infection (Wharton and Lola, 1970). Duchateau and Florkin (1958) reported that the amino acid concentration in haemolymph ranged from 293.3 mg to 2430.1 mg per 100 ml plasma and that tyrosine was present in concentrations up to 230.3 mg per 100 ml plasma. The range of human blood plasma tyrosine is 0.2 to 2.5 mg per 100 ml (Stein and Moore, 1954).

Like other amino acids tyrosine and its metabolic precursor, phenylalanine, are essential for protein and peptide biosynthesis. This role is a vital one as proteins function as enzymes, hormones, antibodies, and structural and contractile units in addition to serving other important but poorly understood functions.

Cross-linking of tyrosine into di- and trimers by a covalent bond in the ortho-position results in resilin, a rubber-like protein capable of storing elastic potential energy, found in the wing hinges and ligaments of some insects. The amino acid sequence in resilin is such that it prevents other cross-links from occurring, since these would impair the rubber-like properties (Anderson, 1964; Anderson and Weis-Fogh, 1964). In the locust between one quarter and one third of the recoil

energy of the wing is due to the elasticity of resilin in the wing-hinge ligament. The peculiar cross-linking system, derived from tyrosine, is the key to this protein's unique properties.

The active constituents of insect chemical defenses are often quinones and other phenolic compounds. Although the biosynthetic pathways of these compounds are relatively unexplored it is assumed that they are derived from phenylalanine and tyrosine (Weatherson, 1967).

Noradrenaline and dopamine have been found to be active on electrophysiological preparations of insect central nervous system (Hodgson and Wright, 1963) and these catecholamines have been isolated from the sub- and supraesophageal ganglia (brains) of cockroaches (Frontali and Häggendal, 1969). Dopamine, the precursor of noradrenaline, is derived from tyrosine in insects and these and other similar compounds are currently under investigation as neurotransmitters and/or hormones in insects (Wigglesworth, 1970).

Tyrosine metabolites are also involved in the hardening and darkening on the insect cuticle. N-acetyldopamine and N-acetyltyramine have been identified as sclerotizing agents in several species by Karlson and his co-workers. Anderson and Barnett (1971) have reported the isolation of ketocatechols from insect cuticle and their possible role in sclerotization.

In Periplaneta americana tyrosine can be hydroxylated to DOPA or decarboxylated to tyramine (Mills et al., 1967) and both of these metabolites can be converted to dopamine (Whitehead, 1969). N-acetylation results in N-acetyldopamine which is further oxidized

to o-quinones. These quinones form phenolic cross-links with the cuticular proteins resulting in the sclerotization of the cuticle. This is the primary pathway of tyrosine in teneral and young adult P. americana (Murdock, 1969; Murdock et al., 1970a; Mills and Lake, 1971). As the adult age of the cockroach increases a shift in this metabolic pathway takes place (Murdock et al., 1970a; Hopkins et al., 1971). A similar shift in metabolic pathways and reduction in decarboxylation was observed in last stage larvae of P. americana throughout the interecdysial period (Hopkins et al., 1971). Tyrosine is decarboxylated to form N-acetyldopamine at a lower rate and this pathway may be replaced by another route, probably similar to the transamination, reduction, beta-oxidation scheme found in Schistocerca gregaria (Karlson and Herrlich, 1965). Although the confirmation of this pathway in older adult P. americana remains to be verified, the work of Murdock (1969) and Mills and Lake (1971) give it substantial support.

This shift in tyrosine metabolism with age has also been reported to occur in several other species: Calliphora erythrocephala (Karlson et al., 1962; Sekeris and Karlson, 1962), Locusta migratoria (Karlson and Herrlich, 1965) and Tenebrio molitor and Drosophila melanogaster (Sekeris and Herrlich, 1966).

Other tyrosine derivatives, protocatechuic acid (Pryor, 1946) and 3,4-dihydroxybenzyl alcohol (Brunet, 1967) serve in the sclerotization of cockroach oothecae.

Melanin, the dark brown to black pigment found in almost all animals, is another tyrosine metabolite. In insects melanin is usually confined to the cuticle (Gilmour, 1965).

In insects tyrosine is derived from two major sources: hydrolysis of exogenous and endogenous proteins and peptides, and hydroxylation of phenylalanine.

Although phenylalanine is an essential amino acid for most insects studied (House, 1965), its metabolic fate has been examined in only a few species. Murdock *et al.* (1970c) lists the insects in which tyrosine is known to be formed from phenylalanine. More recent additions to this list include Calliphora erythrocephala (Schloerer *et al.*, 1970; Price, 1967) and P. americana (Murdock *et al.*, 1970c). This conversion is catalyzed by the enzyme phenylalanine 4-hydroxylase (E.C. 1.99.1.2) and the cofactor requirements are similar to the vertebrate enzyme (Belzecha *et al.*, 1964). The reaction is not reversible. Price (1970) reports that *in vitro* hydroxylation of tyrosine takes place in the fat body of blowfly larvae.

While the number and variety of physiological roles played by tyrosine in insects is generally known, there are many questions concerning the sources, metabolism, and utilization that remain to be answered. To be able to answer many of these questions it will be necessary to do quantitative analyses of phenylalanine, tyrosine, and their metabolites from haemolymph and tissue samples from individual insects. In some instances it will be necessary for the insect to continue its development after the sample has been taken. This

necessitates small sample sizes. With the advent of trimethylsilyl derivation of aromatic compounds and silicone liquid phases for gas liquid chromatography, a rapid and sensitive method is available.

The specific objectives of this thesis were to: (1) adopt methods utilizing GLC and TMS derivatives that would allow quantitative analysis of phenylalanine, tyrosine and their metabolites from individual tissue samples of insects; and (2) determine the concentrations of phenylalanine and tyrosine in haemolymph, fat body, and integument of the American cockroach, Periplaneta americana, during pre- and post-ecdysial stages of cuticle formation and sclerotization.

METHODS AND MATERIALS

Rearing and Maintenance of Cockroaches

Periplaneta americana were reared in large metal wash tubs having a layer of wood shavings in the bottom and an electrical barrier around the sides as described by Wagner et al. (1964). The insects were provided with water and Purina Lab Chow ad lib. The photoperiod regimen was light 7:00 a.m. to 11:00 p.m. and dark 11:00 p.m. to 7:00 a.m. C.S.T. The temperature of the rearing room was kept at 27°C and the relative humidity at 50 per cent.

Experimental Insects

The colonies were checked several times daily and newly ecdysed male insects collected. Only those adult insects whose wings were unexpanded upon collection were used for time periods 'newly ecdysed' to '24 hours post-ecdysis'. Insects to be used within 24 hours were

placed in individual plastic Petri dishes, 10 x 10 x 1.5 cm. The Petri dishes were equipped with aeration holes and small food and water containers. Insects to be used at a time interval greater than 24 hours post-ecdysis were held in screen-topped pint Mason jars with food and water.

The relationship between eye color and molting as reported by Flint and Patton (1959) was used in selecting larvae for the pre-ecdysial time interval experiments.

Eyes of the inter-molt insect are shiny black. As the cockroach undergoes apolysis and approaches ecdysis the eye color changes from black to smoky grey to powder blue. During this period the cuticle becomes progressively softer to the touch. Last instar male larvae were held in plastic Petri dishes and observed daily for a change in eye color.

The 'inter-molt' period refers to that time interval when sclerotization of the new cuticle is relatively complete and before the next molting cycle begins with apolysis. Although endocuticle continues to be formed during the inter-molt period in some insects (Chapman, 1969) it is not sclerotized.

Apolysis refers to the separation of the old cuticle from the underlying epidermal cells and signals the end of the inter-molt period. Scheie (1969) has observed a change in the electrical d.c. resistance across the integument of P. americana during the molting cycle. Changes culminate in a peak within 10 days of the impending molt, and drop to a minimum value immediately following a molt. He associates this

phenomenon with the closing off of channels in the old cuticle which had been used for transport of material between the epidermal cells and the external surface. Another indication of an impending molt in P. americana is the change in eye color (Flint and Patton, 1959) as previously mentioned. Color change is observed after the resistance peak has been reached (Scheie, 1969). The fluid present between the exuvium (old cuticle) and new cuticle turns white on exposure to air. This suggests that the milky appearance of the eyes results from such a reaction taking place under the thin exuvium which has begun to leak air. The white material over the black eye pigment would result in the milk blue appearance (Scheie, 1969).

Although no direct evidence is available, it is assumed that separation of the old cuticle from the epidermal cells (apolysis) and eye color change are correlated. Apolysis as used in Figures 1 and 2 refers to the time interval between eye color change and ecdysis.

Amino Acid Extraction

The extraction solvent consisted of 0.1 N HCl and 2 mM ascorbic acid in methanol. Phenylalanine, tyrosine, and their metabolites are highly soluble and stable in this solvent, and all enzymatic activity in the sample is stopped during homogenization, an essential requirement for accurate amino acid determinations in tissue. Fraenkel and Rudall (1947) found a 20 per cent loss of haemolymph tyrosine after standing at room temperature for 3 hours. The ascorbic acid serves as an anti-oxidant, to minimize degradation of labile metabolites, and also as an internal GLC standard for the calculation of relative retention times.

Acid methanol extractions were made on samples of haemolymph, fat body and abdominal integument. The abdominal integument includes the abdominal cuticle and the underlying epidermal cells.

Blood samples were collected by severing the right meta-thoracic femur and transferring the haemolymph to a preweighted stoppered glass vial, 15 x 45 mm (Kimble Glass Co., Owens, Ill.), with a disposable Pasteur pipet. Immediately after weighing cold extraction solvent was added to give 10 ul solvent per mg haemolymph. The vial was capped and held at 1°C for two hours. An aliquot was taken directly from the vial for silylation.

After collection of the haemolymph sample, the legs, head, and wings were removed and a ventral longitudinal incision was made. The insect was pinned, dorsal side down, in a dissecting dish exposing the body cavity and the entire gut was removed. A fat body sample was collected with forceps and placed on the ground glass portion of a tared pestle for weighing. Remaining fat body and tracheae were cleaned from the abdominal cavity with forceps and blotting tissue. The cleaned integument was cut from the thorax, weighted, and placed into a ground glass mortar tube.

The extraction procedure for fat body and integument samples was identical: after weighting, cold extraction solvent was added to the mortar tube to give 10 ul solvent per mg of wet weight tissue. The tissue was homogenized, the pestle removed, the mortar tube sealed with Parafilm "M" (Matathon Products, Neenah, Wisconsin), and held for 2 hours at 1°C. The homogenate was then transferred to a 50 x 5 mm centrifuge tube constructed from 5 mm (O.D.) heavy walled Pyrex tubing. This

microcentrifuge tube was sealed with a small square of Parafilm and centrifuged at 7000 x g for 15 minutes at 2-4°C. The resulting supernatant was used for silylation.

To check the efficiency of the extraction procedure, tissues from insects 3.3 hours and 24 hours post-ecdysis were treated as follows: haemolymph, fat body and abdominal integument samples were collected and homogenized as previously described. The samples were held for 2 hours at 1°C, transferred to 8 ml heavy walled centrifuge tubes, sealed with Parafilm, and centrifuged. Extracts 'A' were taken from this supernatant. The supernatant was decanted, the pellet washed three times with 300 ul cold methanol per wash and then resuspended in cold extraction solvent, 10 ul solvent per mg original wet weight. This suspension was held 24 hours at 1°C, centrifuged and extracts 'B' taken from the resulting supernatant.

Extracts 'A' and 'B' were silylated, analyzed for tyrosine content and the extraction efficiency calculated as follows:

$$\frac{\text{ug tyr/mg Ext. A} - \text{ug tyr/mg Ext. B}}{\text{ug tyr/mg Ext. A}} \times 100 = \text{Extraction efficiency}$$

The results of the extraction efficiency studies are in Table 1. In all tissues the 2 hour interval extracted over 90 per cent of the total free tyrosine available. In the haemolymph samples the initial extraction was over 99 per cent efficient.

The 24 hour extraction resulted in small additional amounts of tyrosine (see Table 1) Increases in the concentration and total number of GLC peaks present as compared to the 2 hour extraction were also

Table 1. Efficiency of tyrosine extraction from haemolymph, fat body and abdominal integument.

Time post- ecdysis	Tyrosine Concentration--ug/mg wet weight tissue					
	3.3 Hours			24 Hours		
Extract ¹	A	B	EE ²	A	B	EE ²
Haemolymph	2.05	0.10	99.5%	0.40	trace	99+ %
Fat body	6.26	0.46	92.7%	1.04	0.10	99.4%
Abdominal integument	3.80	0.32	91.6%	0.85	0.03	96.5%

¹ Extracts 'A' and 'B' extracted sequentially for 2 and 24 hours, respectively, centrifuged, and supernatant aliquots silylated and analyzed for tyrosine content.

² Extraction efficiency (EE) = $100 \times \frac{\text{ug tyr/mg Ext. A} - \text{ug tyr/mg Ext. B}}{\text{ug tyr/mg Ext. A}}$

observed. In addition to the convenience of a shorter extraction time the 2 hour extraction results in a 'cleaner' extract when examining phenylalanine and tyrosine by GLC.

Due to the low concentrations of free phenylalanine detected and its high solubility in the extraction solvent, an extraction efficiency check for phenylalanine was not conducted and the 2 hour interval was used.

Estimations of total fat body weight per insect were done on newly ecdysed and 24 hour post-ecdysis adult males. The insect was dissected as previously described and the fat body collected with forceps and placed on a tared piece of aluminum foil for immediate weighing. Five insects were used for each time period.

Effects of Haemolymph Sampling on Ecdysis

The average haemolymph sample taken from apolysis stage cockroaches was about 10 mg per insect. This reduction of the haemolymph volume and the trauma of severing a leg of the insect undergoing new cuticle formation could possibly delay or accelerate ecdysis. The haemolymph sampling did not, however, affect the insect's ability to undergo ecdysis, wing expansion, or sclerotization. Sampled and control insects displayed about the same amount of activity during apolysis and post-ecdysial periods. The time interval between sampling and ecdysis ranged from 15 minutes to several days and did not show a clustering effect. This evidence suggests that haemolymph sampling caused a minimal disruptive effect of the apolysis to ecdysis time interval.

Silylation Procedure

Silylation, as used here, refers to the process by which an active proton is replaced by a trimethylsilyl (TMS) group. The silylation reagent donates the TMS group while the compounds to be derivatized must carry an active proton, e.g., hydroxyl, amino, or carboxylic acid.

Extracts to be silylated were transferred from the centrifuge tube to a 15 x 45 mm screw cap vial with teflon-faced septum (Pierce Chemical Co., Rockford, Ill.) with a 50 or 100 ul Hamilton syringe (Hamilton, Reno, Nevada). The extract was evaporated to dryness using low heat and a stream of nitrogen and silylated with a mixture of Regisil \overline{N} , 0, bis-(trimethylsilyl) trifluoroacetamide $\overline{7}$ plus 1% TMCS (trimethylchlorosilane) and silylation grade acetonitrile (1/1) (Regis Chemical Co., Morton Grove, Ill.).

To reduce contact with atmospheric water the silylation mixture was made up in an 1 ml Hamilton gas tight syringe. After equal parts of Regisil plus 1% TMCS and acetonitrile are drawn up into the syringe and mixed, the needle was removed and the silylation mixture transferred to sample and standard vials through the orifice in the luer tip with a 100 ul syringe.

The volume of silylation mixture used depended on the tyrosine or phenylalanine concentration of the extract as determined by pilot studies. For extracts with high amino acid concentration 100 ul was used; extracts with lower tyrosine or phenylalanine concentrations were silylated with 50 ul of the mixture. The volume of extract silylated ranged from 50 to 100 ul.

After addition of the silylation mixture the vial was heated at 85°C for 15 minutes and then allowed to cool to room temperature before use. GLC samples were drawn through the septum. Usual sample size injected was 2-4 μ l. The same procedure was followed for silylation of the tyrosine and phenylalanine standards previously dissolved in the extraction solvent.

Silylation of tyrosine and phenylalanine was found to be complete after 15 minutes at 85°C. This agrees with the results listed in the Regis catalog (Regis Chemical Co., 1972, p. 19, Chicago, Ill.) which shows silylation of tyrosine to be relatively complete after 20 minutes at room temperature when reacted with a mixture of Regisil-acetonitrile (1/1).

Gas-liquid Chromatography

The gas-liquid chromatography (GLC) procedure used is similar to that of Atkinson *et al.* (1971). Six feet by 1/4 inch (O.D.) silanized glass columns packed with either 7% OV-1 or 5% OV-17 (Pierce Chemical Co.) on Gas Chrom Q (80/100) (Applied Science Laboratories, Inc., State College, Penn.) were used. The OV-1 columns were used in a Research Specialties Co. GLC utilizing a hydrogen flame detector. The OV-17 columns were used in a Barber Coleman Model 10 GLC, equipped with an argon ionization detector. An Autolab 6300 digital integrator (Mountain View, Calif.) was used with both instruments for relative peak area and retention time determinations. Gill and Toa (1965) reported that peak quantitation using a digital integrator resulted in the best relative precision of the six methods tested.

Coating of the solid support was carried out as follows. A weighed amount of OV-1 was dissolved in hot toluene in a large crystalizing dish (170 x 90 mm). Gas-Chrom Q giving a 7% (w/w) coating was added and the suspension stirred as the solvent evaporated. Some clumping was observed but clumps were removed by sifting before column packing. Attempts to coat the Gas-Chrom Q using a rotary evaporator procedure failed because evaporation of the solvent resulted in foaming. This problem was not encountered with the OV-17 and a rotary evaporator procedure was used. Distilled acetone was used as the solvent.

The GLC columns were silanized as follows: Borosilicate glass columns were filled with acid cleaning solution and left overnight. After a distilled water wash, a water aspirator was attached to the empty column and 300 ml of chloroform followed by the same volume of acetone was drawn through the column. The column was then flushed with 300 ml of acetone, the inlet blocked and vacuum continued until the column was dry. A fresh solution of dimethyldichlorosilane (Pierce Chemical Co) in toluene (10% v/v) was poured into the column and allowed to stand for 10 minutes. This solution was then poured out and the column rinsed with 300 ml of toluene using the vacuum aspirator. The column was then disconnected from the vacuum, filled with methanol, and allowed to stand quietly for about 5 minutes. After draining, the column was connected to the vacuum again and rinsed with 50-100 ml of methanol. A check of the methanol was made with pH paper to insure neutrality. A final acetone rinse permitted rapid drying of the column. The columns were always packed immediately after silylation.

A hand vibrator was used to settle the packing material in the column and silanized glass wool plugs inserted into the inlet and outlet.

Columns were conditioned overnight or longer at 250°C with a carrier gas flow of 5-10 ml/min. During the conditioning the column was disconnected from the detector.

Column performance and on-column degradation of silylated compounds varied with column age. Daily injections of "Silyl 8" column conditioner (Pierce Chemical Co.) reduced or eliminated on-column breakdown and seemed to prolong column life. Atkinson (personal communication) recommends several large injections of DOPA in acetonitrile-BSA (N,O-bis(trimethylsilyl)-acetamide) before each series of runs to greatly improve the response of amino acid derivatives.

It was necessary to clean the hydrogen flame detector of silicon build up every 100 hours of operation time to retain sensitivity. Cleaning was done with acetone and a small test tube brush.

The parameters of the two gas chromatographs are listed in Table 2. Chart speeds of both recorders were 15 inches per hour. A cell voltage of 800 volts was used with the argon ionization detector.

Standard Compounds

All of the standards used, except N-acetyldopamine, were purchased from the following commercial sources: L-phenylalanine, dopamine hydrochloride, and 3,4-dihydroxybenzoic acid (protocatechuic acid), Mann Research Laboratories, New York, New York; p-hydroxyphenylacetic acid, p-hydroxyphenyl propionic acid (phloretic acid), 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxyphenylcinnamic acid, K & K Laboratories, Inc.,

Plainview, New York; L-tyrosine and *p*-hydroxyphenylpyruvic acid, Nutritional Biochemical Corporation, Cleveland, Ohio; tyramine hydrochloride and *p*-hydroxyphenylcinnamic acid, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; L(+) ascorbic acid and DL-beta-3,4-dihydroxyphenylalanine (DL-DOPA), Matheson Coleman & Bell, Norwood, Ohio and Sigma Chemical Company, St. Louis, Missouri, respectively.

N-acetyldopamine was synthesized according to Mills *et al.* (1967). Although the light brown product could not be crystalized, the IR spectrum (KBr pellet) compared favorably with that in the literature (Karlson *et al.*, 1962). TMS derivation of the product and GLC analysis resulted in a single symmetrical peak on the 7% OV-1 column. The relative location of the *N*-acetyldopamine derivative on OV-1 is in agreement with that reported by Atkinson *et al.* (1971).

Retention Time of Standards

The retention time (R_t), in minutes, and the relative retention time (R_t') using ascorbic acid as the internal standard are listed in Table 2. Relative retention times are listed as a fraction of the retention time of ascorbic acid. The use of R_t' 's compensates for daily fluctuations of the GLC parameters.

It was necessary to use the OV-17 column to check for *p*-hydroxyphenylpropionic acid (pHPPrA), a known tyrosine metabolite in insects (Karlson and Sekeris, 1962; Mills and Lake, 1971), because it and phenylalanine have the same R_t' on OV-1.

Table 2. Retention times (R_t), in minutes, and relative retention times (R_t') for trimethylsilyl (TMS) derivatives of phenylalanine, tyrosine and possible metabolites.¹

TMS Derivative	OV-1		OV-17	
	R_t	R_t'	R_t	R_t'
<u>p</u> -hydroxyphenylacetic acid	solvent peak ²		----	----
phenylalanine	3.86	0.24	4.41	0.23
<u>p</u> -hydroxyphenyl propionic acid	3.86	0.24	6.38	0.34
tyramine	13.15	0.82	----	----
<u>p</u> -hydroxyphenyl cinnamic acid	13.29	0.83	----	----
tyrosine	14.43	0.90	----	----
ascorbic acid	16.09	1.00	18.90	1.00
<u>N</u> -acetyldopamine	20.63	1.28	----	----
<u>p</u> -hydroxyphenyl pyruvic acid	22.65	1.41	----	----
dopamine	22.80	1.67	----	----
DOPA	29.10	1.81	----	----

¹ Ascorbic acid was used as the internal standard for calculation of relative retention times. Columns used with respective detector and parameters are as follows: The 7% OV-1 on Gas Chrom Q (80/100) column was run isothermally at 200°C in conjunction with a hydrogen flame detector. Flow rates were: nitrogen-45 ml/min, hydrogen-32 ml/min, and air-250 ml/min. The 5% OV-17 on Gas Chrom Q (80/100) column was run isothermally at 175°C in conjunction with an argon ionization detector. Argon flow rate was 50 ml/min. Both columns were glass, 1/4 inch (O.D.) by 6 feet.

² p-Hydroxyphenylacetic acid had a R_t of 2.91 minutes on OV-1 at 175°C.

Only small quantities of a compound with the same R_t as pHPPrA on OV-17 (0.29) were detected in the extracts. The presence of pHPPrA in these extracts would agree with the work of Mills and Lake (1971) who reported in vitro synthesis of this compound from tyrosine by haemolymph enzymes from newly ecdysed larvae, intra-ecdysial larvae, and adult female P. americana.

TMS derivatives of known tyrosine metabolites were also checked to insure that these compounds did not interfere with GLC analysis of phenylalanine and tyrosine. In addition to the metabolites listed in Table 2, the following dihydroxyphenolic acid compounds were checked: 3,4-dihydroxyphenyl acetic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid) and 3,4-dihydroxycinnamic acid. No interference was observed.

Evaluation of GLC Data

Standard curves were run with each set of silylated extracts. Injection of standards was done in triplicate and averaged to obtain values for standard curve construction. Results were plotted as ug amino acid vs. relative peak area calculated by the digital integrator. A linear relationship was observed in the range of the concentration used.

GLC injections of silylated extract aliquots were also done in triplicate and relative peak area values averaged. These values were plotted on the respective standard curve and experimental phenylalanine or tyrosine values calculated as ug amino acid per mg wet weight tissue. Figures 1 and 2 were constructed by plotting ug amino acid per mg wet weight tissue vs. time pre- and post-ecdysis. Each point on these figures is the

average of four or more values from individual insects. Standard error of the mean was calculated for each point to give an estimation of variability.

RESULTS

Phenylalanine Concentrations

Phenylalanine is a known precursor of tyrosine in P. americana (Murdock et al., 1970c). For this reason it is necessary to consider the role of phenylalanine when studying tyrosine metabolism and utilization for sclerotization. The free phenylalanine concentrations in P. americana haemolymph during apolysis and post-ecdysis are shown in Figure 1. There was no significant change in phenylalanine concentrations of about 0.30 ug per mg haemolymph during apolysis to ecdysis. However, at 5-10 days post-ecdysis this value drops to about 0.18 ug phenylalanine per mg haemolymph.

A phenylalanine haemolymph value for last stage larvae was obtained prior to apolysis using the OV-1 column, and it is possible that this value includes some p-hydroxyphenylpropionic acid. Because of this the value obtained represents the upper concentration limit only. This value, 0.90 ug phenylalanine per mg haemolymph, does not appear in Figure 1.

Phenylalanine concentrations in the fat body and abdominal integument during the teneral period when sclerotization and pigmentation of the cuticle are occurring is shown in Figure 2. These values, about 0.25 ug per mg wet weight tissue for both, show no significant change in the

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Figure 1. Phenylalanine and tyrosine concentrations in male Periplaneta americana haemolymph pre- and post-larval-adult ecdysis. Vertical lines are twice the standard error of the mean.

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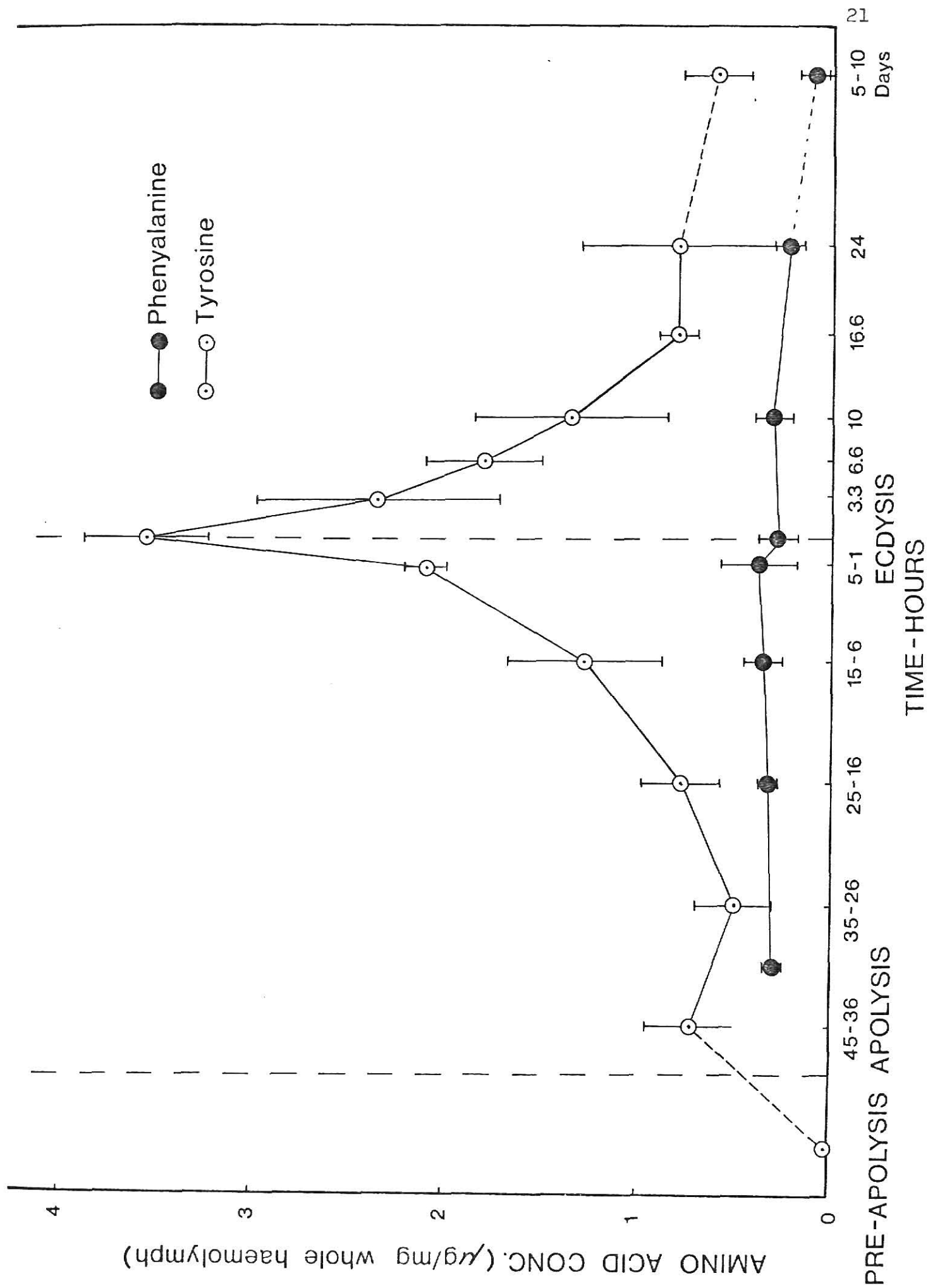
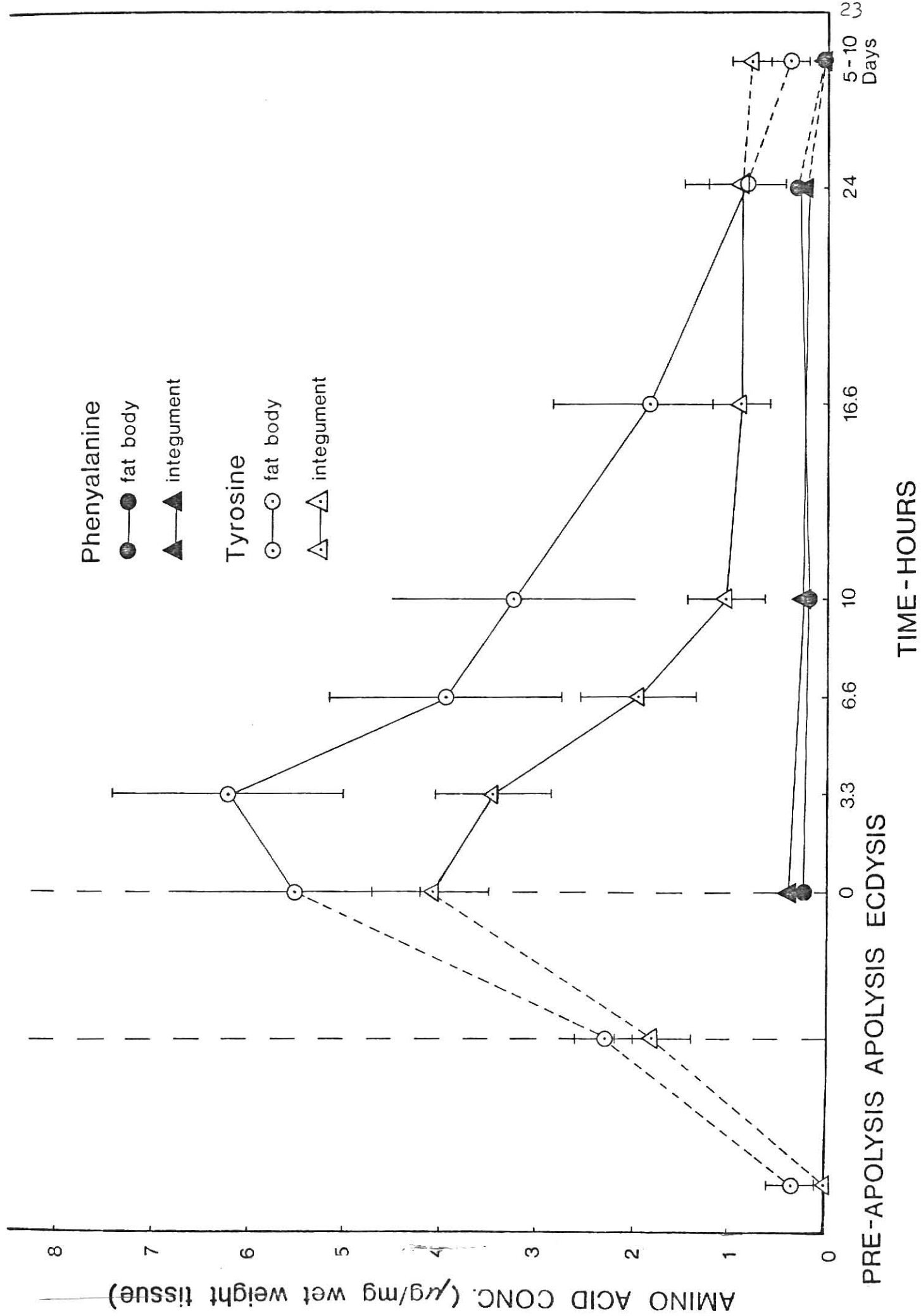


Figure 2. Phenylalanine and tyrosine concentrations in male Periplaneta americana fat body and abdominal integument pre- and post-larval-adult ecdysis. Vertical lines are twice the standard error of the mean.



concentration for the first 24 hours post-ecdysis. This level drops to about 0.06 ug per mg tissue at 5-10 days post-ecdysis.

Analysis of free phenylalanine concentrations for the pre-apolysis time interval were done on the OV-1 column and therefore not included in Figure 2 since they may include some p-hydroxyphenylpropionic acid. The upper limit values for fat body and abdominal integument are 0.69 ug and 1.22 ug phenylalanine per mg tissue, respectively.

Tyrosine Concentrations

The pre- and post-ecdysial free tyrosine concentrations for haemolymph are shown in Figure 1. Free tyrosine in the haemolymph of the cockroach prior to the formation of a new cuticle is very low, i.e., about 0.05 ug per mg. With the onset of apolysis the haemolymph concentration of free tyrosine increases over ten fold. This level fluctuates little during early apolysis but at 16-25 hours pre-ecdysis the tyrosine concentration again begins to increase, reaching its highest level at ecdysis; 3.5 ug per mg haemolymph. This is a 70 fold increase over the pre-apolysis value. The tyrosine concentration then drops rapidly until about 16.6 hours post-ecdysis where it again plateaus at 0.60-0.80 ug free tyrosine per mg haemolymph through 5-10 days post-ecdysis.

Pre-ecdysial and post-ecdysial free tyrosine values for fat body and abdominal integument are shown in Figure 2. Since it was necessary to sacrifice the insects in order to take these samples, it is impossible to know the exact time interval before ecdysis would have occurred. Tissues from black-eyed males, 15-20 days after the last larval molt were used for the pre-apolysis samples.

Apolysis samples were taken from blue-eyed, male larvae about 24 hours after the initial eye color change. Haemolymph samples taken from these same insects show a free tyrosine concentration at 1.06 ug per mg haemolymph. Comparing this value with the pre-ecdysial tyrosine values in Figure 1, the insects used for fat body and integument analysis were in the range of 16 to 25 hours pre-ecdysis.

Free tyrosine concentrations for fat body and abdominal integument during apolysis were 2.32 ug and 1.77 ug per mg wet weight tissue, respectively. These values increased to 5.47 ug and 4.09 ug per mg tissue, respectively, at ecdysis.

The free tyrosine concentrations in the fat body and abdominal integument exceed the respective haemolymph value during both apolysis and ecdysis.

The continued increase in fat body free tyrosine content at 3.3 hours post-ecdysis was not statistically significant (Figure 2). The decrease in free tyrosine post-ecdysis in both fat body and abdominal integument is similar to the decrease observed in the haemolymph. The integument values, however, decrease at a higher rate, leveling off at about 10 hours post-ecdysis. The fat body free tyrosine shows a more gradual slope and continues to decrease 5-10 days post-ecdysis. The haemolymph free tyrosine concentrations leveled off at about 16.6 hours post-ecdysis (Figure 1).

Fat Body Estimations

The total fat body of newly ecdysed and 24 hour post-ecdysial adult, male P. americana was 86.4 (\pm 19.9) mg and 95.3 (\pm 23.5) mg per insect, respectively.

DISCUSSION AND CONCLUSIONS

This study has shown that in the haemolymph, fat body and integument of P. americana the free tyrosine concentration rises between apolysis and ecdysis and then proceeds to decrease during cuticle sclerotization. It is assumed that this tyrosine is utilized after ecdysis for the hardening and darkening of the cuticle. This is the first report on the tyrosine concentrations in individual tissues of a hemimetabolous insect throughout metamorphosis and the sclerotization of the cuticle.

Most studies reported tyrosine concentrations in haemolymph samples or whole extracts from holometabolous insects, and primarily Cyclorrhaphous Diptera in respect to puparium formation, a rather specialized form of sclerotization.

As reported here the increase in haemolymph tyrosine in P. americana between apolysis and ecdysis takes place in two stages. There is an initial increase at apolysis with the concentration remaining at this level until about 20-30 hours pre-ecdysis. A second much sharper rise then begins and peaks at ecdysis. The tyrosine concentration then falls rapidly for about 16.6 hours post-ecdysis, after which it remains relatively constant for 5-10 days after the molt.

In Phormia regina (Levenbook, 1966) haemolymph tyrosine builds up during early and last larval instar and decreases sharply prior to pupation. Calliphora erythrocephala (Price, 1970, 1972) and Bombyx mori (Duchateau-Bosson et al., 1962) show a sharp rise in haemolymph tyrosine during the late last larval stage and a rapid decline with puparium hardening and darkening. In Rhodnius prolixus (Price, 1972), the only

hemimetabolous insect examined previously, such rapid changes are not observed. The tyrosine concentration rises gradually attaining a maximum level at the larval-adult ecdysis, after which it slowly declines.

While the decrease in post-ecdysial haemolymph tyrosine in P. americana is similar to that reported in other insects, the pre-ecdysial levels, with the increase in two distinct phases has so far not been observed in other insects. It must be stressed, however, that with the exception of the blood feeding hemipteran R. prolixus, P. americana is the only hemimetabolous insect so far examined.

In some insect species specific tissues appear to serve as storage organs for the tyrosine used during sclerotization. Price (1967, 1969, 1970, 1972) has done extensive work on the role of fat body as a tyrosine synthesizing and storage organ in C. erthycephala. Of the total free tyrosine (approximately 275 ug) in the third-instar larva, at least 80 per cent is present in the fat body and 7 per cent in the haemolymph. At the commencement of puparium formation tyrosine is rapidly released from the fat body into the haemolymph (Price, 1972).

Unlike the fat body of *Calliphora*, which acts as a relatively long term storage organ for free tyrosine, the fat body of P. americana begins to increase in tyrosine at apolysis. Since this increase in fat body tyrosine occurs simultaneously with haemolymph tyrosine it would indicate that free tyrosine is not stored in this tissue prior to apolysis but is formed from some other precursor.

One possible source of the free tyrosine that accumulates is biosynthesis from phenylalanine. Although phenylalanine is an essential

amino acid for P. americana, our results show that only a minor pool of free phenylalanine is available in haemolymph, fat body and integument tissues for hydroxylation to tyrosine during this time. From these data it would appear that pre-ecdysial free phenylalanine plays no more than a minor role as a metabolic source of tyrosine during this time. Murdock et al. (1970b), however, reported that in P. americana injected with L-phenylalanine-1-C¹⁴ or -U-C¹⁴ shortly after ecdysis, more than half of the radioactivity appeared as tyrosine after 24 hours, indicating extensive conversion. Since part of the tyrosine side chain is known to be oxidized (Murdock et al., 1970a), this biosynthesis was even more extensive than was indicated by these experiments. During the same time interval we found no significant change in haemolymph phenylalanine levels of P. americana.

A mechanism that could support both sets of data has been reported by Bodnaryk (1970a). He reports the presence of a dipeptide, gamma-L-glutamyl-L-phenylalanine, that is synthesized by Musca domestica during larval growth. The concentration of this dipeptide reaches a maximum level in the fully grown larva, decreases rapidly during the first few hours of sclerotization, and cannot be detected 8 hours after the start of cuticular tanning. During this time there is virtually no change in the levels of free glutamic acid and phenylalanine. It was suggested that gamma-L-glutamyl-L-phenylalanine plays a key role in puparium formation by providing a large phenylalanine reservoir for metabolism to melanin and/or quinones via tyrosine to be utilized during the sclerotization process. Although gamma-L-glutamyl-L-phenylalanine has been detected only in the genus Musca, no hemimetabolous species were reported checked.

The build up of gamma-L-glutamyl-L-phenylalanine during larval growth followed by its rapid metabolism at pupation follows the same pattern as tyrosine-0-phosphate in Drosophila melanogaster (Lunan and Mitchell, 1969) and beta-alanyl-L-tyrosine (sarcophagine) (Bodnaryk and Levenbook, 1969) in Sarcophaga bullata. Both of these compounds are thought to serve as tyrosine reserves for puparium sclerotization. While tyrosine-0-phosphate has been detected only in Musca, only holometabolous species were reported checked. Bodnaryk (personal communication) states that there seems to be no trace of beta-alanyl-L-tyrosine in P. americana.

The existence of low molecular weight compounds having a high tyrosine content in Drosophila and Sarcophaga suggests another possible source of the free tyrosine build up in P. americana prior to sclerotization. Murdock (1969) reports the presence of two tyrosine containing peptides in methanol extracts of young adult male P. americana, injected 12 hours earlier with ^{14}C -labeled tyrosine. Both chromatographic peaks were present when L-tyrosine-1- ^{14}C was injected, indicating the presence of the carboxyl carbon. This would be the case if these peaks were peptides containing tyrosine.

Unchanging levels of phenylalanine in fat body and haemolymph during metamorphosis indicate a steady state of phenylalanine production from storage sources balancing conversion of tyrosine.

While insects are not capable of synthesizing phenylalanine many species harbor symbiotic bacteria in the fat body, as well as micro-organisms in the alimentary tract, that do have this ability (Henry, 1962; Lipke et al., 1965). Cockroaches contain symbiotic bacteria in fat body cells termed mycetocytes (Gier, 1947). These organisms appear to be

essential for the normal hardening and/or darkening of some insects. Sannasi (1968) reports that histochemical examination of the cuticle of Reticulitermes assamenis indicated that the lighter color of the defaunated insects is due to the absence of exocuticle formation. The dietary aromatic amino acids in R. assamenis appear to be insufficient and must be supplemented by symbiotic products before adequate amounts of phenolic precursors for sclerotization of the cuticle can be synthesized.

Wharton and Lola (1970) reported that tyrosine was reduced to 41 per cent of normal, but that phenylalanine was not greatly reduced in aposymbiotic American cockroaches. Lysozyme injections were used to destroy the intra-cellular symbionts. In general, the authors were very skeptical of attributing any of the changes observed in the insects to the absence of symbionts because of the side effects of the lysozyme. However, they did state that the lack of pigmentation observed in the progeny of all sterilized insects, regardless of the mode of sterilization, is caused by a seemingly specific process which has been brought about by the absence of the symbionts. The symbionts and microorganisms also supply the insects with important nutritional materials, the lack of which may indirectly affect normal hardening and darkening of the cuticle.

In the present study, tyrosine levels in the integument begin to rise during apolysis and peak at ecdysis similar to haemolymph and fat body tissue. This tyrosine may arise from several sources including digestion of the old endocuticle or transport from the haemolymph and fat body.

Several authors have reported high tyrosine concentrations in the soluble cuticle proteins examined (Hackman, 1953, 1956; Karlson, 1960; Trim, 1941). Since the endocuticle is degraded by proteolytic enzymes and absorbed by the epidermal cells after apolysis, this appears to be an ideal storage mechanism for tyrosine. Arguments against such a mechanism include the reasoning that one should see an increase in the concentrations of all of the amino acids that made up the digested endocuticle. The fact that these other amino acids do not appear in the haemolymph could be explained by the presence of specific carrier molecules that transport the tyrosine through the epidermal cells and into the haemolymph. Koeppe and Gilbert (1973) have presented immunochemical evidence for the existence of proteins that function in transporting dopamine (or a dopamine metabolite) from the haemolymph into the cuticle, and Price (1972) reports that preliminary experiments have indicated that tyrosine may be bound to protein in the haemolymph of P. americana. Neither of these reports have been confirmed as yet.

While the midgut serves as a source of phenylalanine and tyrosine during digestion it is unlikely that increases in alimentary tract digestive activity could account for the rapid rise in tyrosine concentrations that appear between apolysis and ecdysis in P. americana. The role of the alimentary tract in the gradual sequestering and/or synthesis of tyrosine and/or phenylalanine for storage in tissues is probably restricted by the supply of these amino acids in their diet. Duchateau-Bosson et al. (1962) conclude from their studies on B. mori that tyrosine which comes from food sources is probably incorporated into the tissues

and that some tyrosine is also synthesized from phenylalanine. These storage tissues then supply the haemolymph with free tyrosine for metabolism and/or transportation. Digestion of food proteins and peptides and assimilation of the amino acids through the midgut is probably the major source of free tyrosine in insects during the inter-molt periods.

Wheeler (1963) and Mills and Whitehead (1970) have reported that haemolymph volume in P. americana rises during apolysis, reaches a maximum level at ecdysis and declines after ecdysis. This increase would facilitate the splitting of the old cuticle since the increased volume would allow more pressure to be exerted on the old exoskeleton. The increase in volume would also necessitate a far greater rise in tyrosine to account for the much higher concentration in the haemolymph at this time. Dry weight values would undoubtedly show a much sharper rise in haemolymph tyrosine concentration than wet weight values in Figure 1.

Mills and Whitehead (1970) reported values of 178 ul, 242 ul and 172 ul haemolymph per cockroach for pre-apolysial, ecdysial and 24 hour post-ecdysial insects, respectively, using a C¹⁴-carboxyl inulin isotope dilution technique. Wheeler (1963) found somewhat lower values of 138 ul, 159 ul and 114 ul haemolymph per cockroach for the same respective time intervals using an amaranth dye method for the volume determinations. Neither author mentioned the sex of the insects used.

Using these values for haemolymph volume and the tyrosine values reported here of 0.34 ug, 3.56 ug and 0.82 ug tyrosine per mg haemolymph for the same respective time intervals, the total free tyrosine concentrations

of the insect haemolymph were calculated. The values of Mills and Whitehead (1970) yield total haemolymph tyrosine concentrations of 60.5 ug, 861.5 ug and 141 ug tyrosine per insect at pre-apolysis, ecdysis and 24 hours post-ecdysis, respectively. Wheeler's values (1963) calculated to 46.9 ug, 566.0 ug and 93.5 ug haemolymph tyrosine per insect for the same respective time intervals. Using these values the total haemolymph free tyrosine concentration increases 519 to 801 ug from pre-apolysis to ecdysis and drops between 473 and 721 ug tyrosine per cockroach from ecdysis to 24 hours post-ecdysis.

The total fat body weights per insect reported here were 86.4 mg and 95.3 mg for newly ecdysed and 24 hours post-ecdysial insects, respectively. Using these values and the fat body tyrosine concentrations of 5.47 ug and 0.87 ug tyrosine per mg fat body (Figure 2) for the same respective time intervals the average total fat body tyrosine concentration per insect was determined. These values calculated to 472.6 ug and 82.9 ug of fat body tyrosine per newly ecdysed and 24 hour post-ecdysial insects. During this time interval the total free fat body tyrosine decreases by approximately 390 ug per insect. The average haemolymph tyrosine decrease for the same time interval was 660 ug per insect.

These results indicate that between ecdysis and 24 hours post-ecdysis, the time of maximum sclerotization, over 1000 ug of free tyrosine from the haemolymph and fat body is utilized per insect. This is a static value and does not include the tyrosine present in other tissues or that which is transported and/or metabolized in the haemolymph and fat body during this time.

Price (1972) reports the solubility of tyrosine in 1.15 per cent KCl at 25°C as 29 mg per cent. The haemolymph and fat body free tyrosine concentrations in P. americana at ecdysis exceed this solubility by over twelve and eighteen fold, respectively. Several other authors have also reported insect tyrosine concentrations that appear to exceed the solubility limit for this amino acid (Bodnaryk and Levenbook, 1969; Finlayson and Hamer, 1949; Levenbook, 1950; Levenbook et al., 1969; Price, 1972).

One might expect the tyrosine to be present in solid form at these levels; however, no crystals were observed by microscopic examination of P. americana fat body smears. Price (1972) reports that crystals were not discernible in C. erythrocephala fat body that contained tyrosine exceeding its solubility in 1.15 per cent KCl by thirty-three times. However, Rizki and Rizki (1959) and Henderson and Glassman (1969) have reported the presence of crystals and macromolecules, respectively, in D. melanogaster that are associated with storage of tyrosine metabolites.

At present the manner in which the large amount of tyrosine is rendered soluble is unknown.

In their studies on the hormonal control of tanning in P. americana Mills and Whitehead (1970) found that antidiuretic hormone is responsible for the increase in haemolymph volume prior to ecdysis. Comparing their data with the increase in haemolymph tyrosine shows an interesting correlation. Prior to two days pre-ecdysis the antidiuretic hormone is present in low concentrations and the haemolymph volume and tyrosine concentrations have not begun their increases. At two days pre-ecdysis the antidiuretic hormone displays a three fold increase in its former

activity and the haemolymph volume and tyrosine concentrations have begun to increase. At one day pre-ecdysis the antidiuretic hormone has increased over five fold from its initial value and the haemolymph volume is near its highest level. At about this same time, 20-30 hours pre-ecdysis, the haemolymph tyrosine concentration begins the second stage of its increase.

From this evidence it appears that a hormone, perhaps the antidiuretic hormone, is responsible for the changes in the free tyrosine content of the haemolymph. Since values from only one time interval were obtained during the apolysis to ecdysis time interval the concentration of tyrosine in fat body and integument in respect to hormone titer are unknown.

How such a hormone would bring about tyrosine increases in P. americana is speculative. Perhaps hormonal activation of specific genes results in enzyme synthesis that initiates increased free tyrosine production or liberation. A mechanism similar to this has been reported in Calliphora by Karlson and his colleagues. According to this theory ecdysone exerts a specific action upon a gene locus which induces the formation of a specific RNA-polymerase. The messenger-RNA produced passes into the cytoplasm and initiates the synthesis of the key enzyme, dopa-decarboxylase, necessary for N-acetyldopamine and subsequent puparium formation (Wigglesworth, 1970).

Or perhaps the enzyme produced or activated is similar to the dipeptidase that liberates tyrosine from beta-alanyl-L-tyrosine (sarcophagine) in *Sarcophaga* (Bodnaryk, 1970b) prior to puparium

sclerotization. These enzymes could already be present in an inactive form or present in an active form but separated from their substrate by a membrane barrier. In such cases the hormone could initiate free tyrosine production and/or liberation by removal of the enzyme inhibitor or increasing membrane permeability. This later method resembles the process outlined by Mills and co-workers in P. americana where diuretic hormone selectively increases tyrosine permeability to haemocytes. The active enzymes are located within the blood cells and metabolism of tyrosine is initiated by the permeability change.

After ecdysis the tyrosine concentrations in the tissues studied, with the possible exception of fat body, begin to decrease immediately. Fox and Mills (1971) found that labeled tyrosine incorporation into the cuticle continued at a steady rate for about eight hours after ecdysis. Using radiorespirometric techniques and C^{14} -labeled tyrosine, Hopkins et al. (1971) and Murdock (1969) found that in adult male P. americana during the first day after ecdysis about 52 per cent in 700 minutes and 68 per cent in 1500 minutes, respectively, of the injected tyrosine is decarboxylated indicating high decarboxylase activity during this time. The results reported here indicate that tyrosine is removed from the haemolymph at a rapid rate for approximately 17 hours after ecdysis; from the fat body for over 24 hours and from the abdominal integument for about 10 hours post-ecdysis. These data indicate that tyrosine and/or metabolites are transported into the cuticle for several hours and that the sclerotization process may take place over an extended period. This correlates well with the color changes observed in P. americana after

ecdysis. The change from pure white to mahogany brown takes place gradually and an insect 24 hours after ecdysis can still easily be selected from older adults due to its lighter color.

Mills and Whitehead (1970) indicate that the initial steps in the synthesis of N-acetyldopamine take place in the haemocytes and that this synthesis is stimulated by bursicon containing extracts of the terminal abdominal ganglia. They go on to correlate the activity vs haemolymph volume post-ecdysis and the molecular weight of diuretic hormone and bursicon (tanning hormone) and postulate that these hormones are one and the same. According to their hypothesis the diuretic hormone selectively increases the permeability of the haemocytes to tyrosine. In the blood cell the tyrosine is decarboxylated and hydroxylated (or vice versa) to dopamine. Dopamine could then either be N-acetylated in the haemocyte or epidermis. The fact that dopamine is readily permeable to the epidermal cell membrane and its penetration is enhanced by partially purified diuretic hormone supports the latter course (Mills and Whitehead, 1970).

The presence of high concentrations of free tyrosine in the integument (Figure 2) does not support this hypothesis and is in agreement with earlier studies published by Mills et al. (1967) stating that the raw materials for sclerotization are already present in the integument at ecdysis. Mills (1966) has suggested that cockroach bursicon is not concerned with the synthesis of N-acetyldopamine at ecdysis because the hormone is not released until after ecdysis. This is in sharp contrast to the findings of Fraenkel and Hsiao (1965) who state that bursicon is

present in the haemolymph of P. americana at the time of emergence, and in the ganglia and corpora cardiaca at all times.

Using radiorespirometric techniques and C^{14} -labeled tyrosine, Hopkins et al. (1971) found decarboxylation to be extremely low in insects injected 8-24 hours pre-ecdysis and highest at or shortly after ecdysis, indicating that decarboxylase activity is not elevated until this time.

From these data it appears that bursicon, or some similar hormone, active at or shortly after ecdysis, initiates the decrease in free tyrosine in the haemolymph, fat body and integument of P. americana. The possible modes of activation for this hormone are the same as those discussed earlier.

Just as intriguing as the presence of a hormone and its mode of action in initiating free tyrosine increases and decreases is the activation of this hormone itself.

One possible activation mechanism would be the change in physical parameters within the insect. Perhaps it is similar to the system in Rhodnius where stretch receptors in the abdomen, stimulated by a large blood meal, stimulate neurosecretory cells in the corpus cardiacum to release an 'activation hormone' or brain hormone into the haemolymph. This secretion stimulates the prothoracic glands to produce ecdysone, the molting hormone, which initiates changes in the cells concerned with ecdysis (Wigglesworth, 1970).

Since P. americana is a continuous feeder it is doubtful that molting is initiated in this manner. However, other changes within the

body of the insect such as surface area to weight ratio and internal tissue and/or haemolymph pressure due to the restriction by a rigid cuticle could serve as the activation mechanism.

From the evidence presented it has been assumed that changes in the free tyrosine concentration in P. americana pre- and post-ecdysis involves an activation step which results in the appearance of a hormone and its subsequent effect. Another possibility includes the disappearance or decrease in the titer of a regulating substance already present in the insect as the initiating step. This could be similar to the hormonal control of metamorphosis in which the amount of juvenile hormone present at the time of the molt dictates the life stage.

The results of this research, that free tyrosine levels increase in haemolymph, fat body and abdominal integument of P. americana before ecdysis and decline after ecdysis, are in general agreement with the findings of others who have examined this phenomenon. The source(s) of this tyrosine and the control mechanisms involved in its metabolism await further research. The application of GLC analysis to these studies will aid in their elucidation.

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TYROSINE AND PHENYLALANINE CONCENTRATIONS IN HAEMOLYMPH
AND TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA
AMERICANA (L.) DURING METAMORPHOSIS

by

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The sclerotization of the exoskeleton in many insects requires large quantities of tyrosine, and possibly its metabolic precursor phenylalanine, for producing the quinones necessary for cross-linking and tanning of cuticular proteins. In this study pre- and post-ecdysial free phenylalanine and tyrosine concentrations were measured in the haemolymph, fat body and abdominal integument of the American cockroach, Periplaneta americana.

A gas-liquid chromatographic method utilizing trimethylsilyl derivatives of phenylalanine, tyrosine and their metabolites was used for the analyses. This proved to be a very sensitive and rapid method giving qualitative and quantitative data on extremely small samples of tissue and haemolymph extracts.

Haemolymph free tyrosine increased in two stages; an initial increase near apolysis and a second increase 20-30 hours pre-ecdysis, reaching its highest level at ecdysis: 3.5 ug tyrosine per mg haemolymph. During this time the total haemolymph free tyrosine concentration increased by approximately 660 ug per insect. Fat body and abdominal integument began their free tyrosine increases near apolysis. Fat body levels peaked between ecdysis and 3.3 hours post-ecdysis and abdominal integument levels peaked at ecdysis with maximum concentrations of 5.5 ug and 4.1 ug tyrosine per mg wet weight tissue, respectively. Between ecdysis and 24 hours post-ecdysis, the period of maximum sclerotization, the total free tyrosine in haemolymph and fat body decreased by approximately 600 ug and 390 ug per insect, respectively. The free phenylalanine concentration showed no significant change during this time.

The fat body does not appear to function as a storage organ for free phenylalanine or tyrosine during larval development as it does in some holometabolous insects. The sources of the free tyrosine accumulating in the haemolymph, fat body and integument between apolysis and ecdysis are unknown.

This is the first report of tyrosine concentrations in individual tissues of a hemimetabolous insect with respect to metamorphosis.