

TYROSINE GLUCOSIDE HYDROLASE ACTIVITY IN TISSUES OF *MANDUCA SEXTA* (L.): EFFECT OF 20-HYDROXYECDYSONE*

R. F. AHMED†, T. L. HOPKINS† and K. J. KRAMER‡§

†Departments of Entomology and ‡Biochemistry, Kansas State University, Manhattan, KS 66506
and

§U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of
Agriculture, Manhattan, KS 66502, U.S.A.

(Received 15 December 1982; revised 13 April 1983)

Abstract—When 20-hydroxyecdysone was injected into ligated abdomens from wandering larvae of the tobacco hornworm, *Manduca sexta* (L.), the titres of tyrosine glucoside and tyrosine in the haemolymph decreased and increased, respectively as a result of increased tyrosine glucoside hydrolase activity. Apolysis also occurred and the underlying pupal cuticle became tanned, while in control abdomens none of these events occurred. Tyrosine glucoside hydrolase is found primarily in the fat body and in highest activity in animals undergoing pupal ecdysis. The results support the hypothesis that 20-hydroxyecdysone regulates the build up of free tyrosine by inducing or activating tyrosine glucoside hydroxylase in the fat body. Tyrosine is then released back into the haemolymph for subsequent utilization in pupal cuticle tanning.

Key Word Index: Tyrosine, tyrosine glucoside, β -D-glucopyranosyl-O-L-tyrosine, 20-hydroxyecdysone, *Manduca sexta*, tyrosine glucoside hydrolase, fat body

INTRODUCTION

Tyrosine is conjugated with glucose, phosphate or β -alanine in insects belonging to the orders Diptera and Lepidoptera (Brunet, 1980; Kramer *et al.*, 1980a). These conjugates, which are more soluble than free tyrosine, are primarily synthesized during the last larval feeding period and accumulate to highest levels prior to ecdysis. Tyrosine is then released during ecdysis for the biosynthesis of catechols and quinones used to tan the new cuticle (Mitchell and Lunan, 1964; Lunan and Mitchell, 1969; Levenbook *et al.*, 1969; Bodnaryk, 1970a; 1972; Chen *et al.*, 1978; Kramer *et al.*, 1980a; Ishizaki and Umebachi, 1980; Isobe *et al.*, 1981; Lu *et al.*, 1982). The glucose released from tyrosine glucoside in Lepidoptera may also be used for new cuticle formation (Isobe *et al.*, 1981).

The liberation of tyrosine from its conjugate form prior to cuticle tanning may be under hormonal control in conjunction with other ecdysial events. Hydrolysis of β -alanyl-L-tyrosine was shown to be controlled by ecdysone in the fly, *Sarcophaga bullata*, where a dipeptidase is induced by the hormone (Bodnaryk, 1971; Dunn *et al.*, 1977). In *Musca domestica* the disappearance of γ -L-glutamyl-L-phenylalanine was also under control

of ecdysone, presumably via a dipeptidase (Bodnaryk, 1970b).

β -D-Glucopyranosyl-O-L-tyrosine (tyrosine glucoside) is a storage form for tyrosine in *Manduca sexta* (L.) (Kramer *et al.*, 1980a). This conjugate accumulates in the haemolymph during the feeding and wandering periods of the last larval stadium reaching a maximum level before ecdysis. Its concentration in the haemolymph decreases in late pharate pupae as tyrosine increases (Ahmed *et al.*, 1983). Thus, release of tyrosine from tyrosine glucoside may also be under ecdysteroid control via induction or activation of the tyrosine glucoside hydrolase or β -glucosidase.

We investigated the possible role of ecdysone in controlling the release of tyrosine from tyrosine glucoside during pupal development of *M. sexta*. Abdomens from wandering larvae were isolated by ligatures and injected with 20-hydroxyecdysone. Tyrosine and tyrosine glucoside levels were determined periodically as the abdomens formed pupal cuticle, ecdysed and tanned. We also determined the tissue localization of tyrosine glucoside hydrolase as well as its activity during the larval-pupal transformation.

MATERIALS AND METHODS

Ligation and injection of larvae

Larvae of *M. sexta* were reared on an artificial diet according to Bell and Joachim (1976) at $27 \pm 1^\circ\text{C}$ and a non-diapausing photoperiod of 16 h L:8 h D. Larvae at the beginning of the wandering stage were anaesthetized with carbon dioxide and ligatured with thread between the second and the third abdominal segments (Nijhout, 1976). The anterior portion of the larvae was excised and the ligated area rinsed with 70% ethanol. 20-Hydroxyecdysone (Sigma) was dissolved in 10% isopropanol (1 mg/ml) and

*Contribution No. 82-606-j. Departments of Entomology and Biochemistry, Kansas Agricultural Experiment Station, Manhattan, Kansas 66506. Cooperative investigation between ARS, USDA and the Kansas Agricultural Experiment Station. Supported in part by research grant PCM-8003859 A01 from the National Science Foundation. Correspondence and reprint requests to T. L. Hopkins, Department of Entomology, Kansas State University, Manhattan, KS 66506, U.S.A.

sterilized by passing it sequentially through 0.8 μm , 0.45 μm and 0.2 μm membrane filters (Gelman-Metricol). The hormone solutions were kept refrigerated and were used within five days. Each abdomen two days after ligation was injected with 20-hydroxyecdysone (10 $\mu\text{g/g}$ live wt of isolated abdomen) followed by a second injection (5 $\mu\text{g/g}$ live wt) the following day. The solutions were injected into the base of an abdominal proleg after anaesthesia with carbon dioxide. The abdomens of the control larvae were injected with an equal volume of 10% isopropanol.

Haemolymph tyrosine and tyrosine glucoside extraction and determination

We have previously shown that tyrosine glucoside titres are highest in haemolymph suggesting storage in that tissue (Ahmed *et al.*, 1983). To determine the effect of 20-hydroxyecdysone on titres in isolated abdomens, the haemolymph was collected from an incision in the last abdominal segment, weighed immediately, homogenized in glass tissue grinders with cold 80% (v/v) aqueous methanol (0.1 g/ml) containing 5 mM ascorbic acid, and centrifuged at 12,000 *g* for 15 min at 4–5°C. The residue was washed three times with cold extraction solvent, and the pooled supernatants reduced to 2 ml final volume. Tyrosine and tyrosine glucoside titres were determined by gas-liquid chromatography (GLC) as previously described (Ahmed *et al.*, 1983).

Tyrosine glucoside hydrolase localization and its activity during metamorphosis

To determine the tissue localization of the tyrosine glucoside hydrolase system, haemolymph, fat body, integument and gut tissue preparations were assayed for enzyme activity. Haemolymph was collected as described by Ahmed *et al.* (1983). Its volume was measured and mixed immediately (1:2, v/v) with cold phosphate buffer [0.1 M KH_2PO_4 and K_2HPO_4 saturated with phenylthiourea (PTU), pH 7.1, 2–5°C] as described by Dunn *et al.* (1977). The samples were homogenized in glass tissue grinders, centrifuged at 13,000 *g* for 30 min at 4°C, and the supernatant stored at –20°C. Fat body, gut and integument were dissected under cold phosphate buffer saturated with PTU, rinsed several times with buffer and weighed in tared glass tissue grinders. The tissues were homogenized in ice-cold buffer (1 mg tissue/2 μl buffer) and were centrifuged at 13,000 *g* for 30 min at 4°C. The supernatant from several insects was pooled and stored at –20°C.

Tyrosine glucoside hydrolase assay

Tyrosine glucoside hydrolase activity was measured by incubating 0.2 ml of tissue supernatant with 0.2 ml of 5×10^{-3} M tyrosine glucoside in 0.1 M potassium phosphate buffer, pH 7.2 saturated with PTU. Tyrosine glucoside was obtained by purification of the natural product from *M. sexta* as described by Kramer *et al.* (1980a). The purity was greater than 98% when analyzed by high performance liquid chromatography. Control incubations contained tissue supernatant boiled for 5 min or supernatant only without substrate. The experimental and control mixtures were incubated in 1 ml conical vials in a shaker bath at 31°C for 1 h. The reaction was stopped by boiling for 5 min, followed by centrifugation at 9,000 *g* for 15 min. Glucose in the supernatant which interferes with tyrosine analysis by GLC was removed by ion exchange chromatography (Adams *et al.*, 1977; Ahmed *et al.* 1983). Aliquots of the supernatant (0.2 ml) were mixed with 0.5 ml of 30% (v/v) acetic acid and passed over 80 mg of Dowex 50W-X2; 200–400 mesh (hydrogen form) packed in a disposable pipette. The tyrosine was eluted with 0.5 ml 2 M NH_4OH . Tyrosine titres were determined by GLC as described by Wirtz and Hopkins (1977). Protein was measured by the method of Bradford (1976) using the Bio-Rad kit. Tyrosine glucoside hydrolase activity was expressed as

nmoles of tyrosine glucoside hydrolyzed per mg of protein per minute. The data were analyzed statistically by Duncan's multiple range test at the 0.05 level.

RESULTS

Tyrosine glucoside hydrolase activity in haemolymph and other tissues

Tyrosine glucoside decreased from a peak concentration a few hours before pupal ecdysis to low levels at about 12 h after ecdysis (Ahmed *et al.*, 1983). The simultaneous rise in free tyrosine would indicate an increase in tyrosine glucoside hydrolase activity during this time. Therefore, the haemolymph, fat body, gut and integument were isolated from newly ecdysed pupae, homogenized with phosphate buffer and assayed for tyrosine glucoside hydrolase activity. Fat body tissue was most active in hydrolyzing tyrosine glucoside with approx five times greater enzymatic activity than the haemolymph or integument (Table 1). Activity was present in the supernatant from a 12,000 *g* centrifugation. The gut tissue had very low levels of activity, about fifty times less than fat body.

Tyrosine glucoside hydrolase activity in fat body

Tyrosine glucoside hydrolase activity was measured in fat body removed from animals prior to and during the larval-pupal transformation (Fig. 1). Activity was very low in fat body tissue of mature feeding larvae (~0.1 nmol tyrosine glucoside hydrolyzed/mg protein/min). Activity increased about 5-fold at the onset of wandering behaviour and remained constant throughout the wandering stage and during development of the pharate pupa. A dramatic rise occurred after tanning had been initiated in the pharate pupal cuticle (brown thoracic bar stage) with a peak of activity at ecdysis of about four times the level in pharate pupae (2 nmol tyrosine glucoside hydrolyzed/mg protein/min). The enzyme activity decreased sharply thereafter as the cuticle tanned and reached its initial low level about one day after ecdysis. These data established that a tyrosine glucoside hydrolase occurs in the fat body at specific times during pupal morphogenesis of *M. sexta*.

Effects of 20-hydroxyecdysone on tyrosine glucoside and tyrosine titres

When hornworm larvae that were collected during wandering behaviour but before the second large release of ecdysone, were ligated between the second and third abdominal segments, the isolated abdomens failed to pupate, while the anterior portion

Table 1. Tissue localization of tyrosine glucoside hydrolase in *Manduca sexta*

| Tissue* | Enzyme activity (nmol tyrosine glucoside hydrolyzed/mg protein/min \pm SEM†) |
|------------|--|
| Fat body | 1.98 \pm 0.27 (71) |
| Haemolymph | 0.35 \pm 0.11 (12) |
| Integument | 0.44 \pm 0.12 (16) |
| Gut | 0.04 \pm 0.04 (1) |

*The tissues were isolated from newly ecdysed pupae.
†SEM = standard error of means, $n = 3$. Percent of total activity given in parentheses.

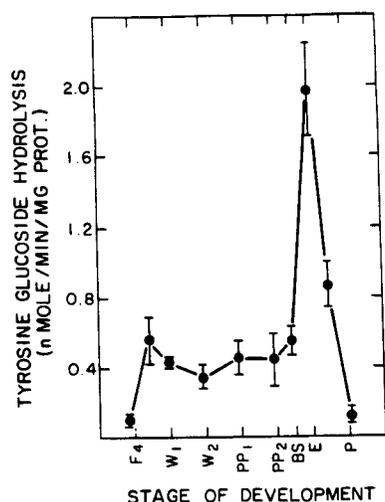


Fig. 1. Tyrosine glucoside hydrolase activity in fat body tissue during larval-pupal development of *Manduca sexta*. F = feeding larva. W = wandering larva. PP = pharate pupa. BS = brown thoracic bar stage. E = pupal ecdysis. P = pupa. Bars = standard error of means, $n = 2-3$.

pupated and its cuticle became tanned. If the release of tyrosine from its glucoside is controlled by 20-hydroxyecdysone, then the abdomens injected with the hormone should show an increase of free tyrosine. In control abdomens injected with solvent only, the titres of tyrosine glucoside and tyrosine in the haemolymph remained at relatively constant levels of 6 and 2 mg/g haemolymph, respectively, during the six days following ligation (Fig. 2A).

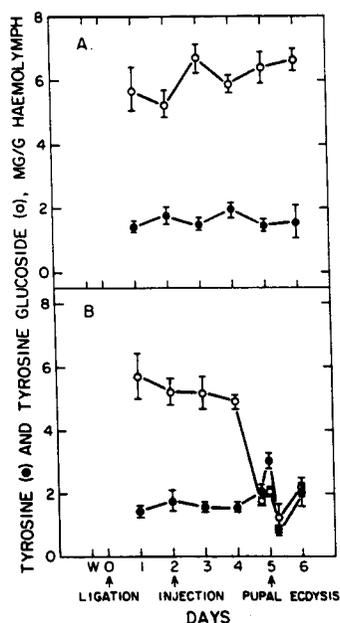


Fig. 2. The effect of 20-hydroxyecdysone on tyrosine and tyrosine glucoside titres in haemolymph of isolated larval abdomens of *Manduca sexta*. (A) solvent injected control. (B) abdomens injected with 20-hydroxyecdysone. W = wandering stage. Bars = standard error of means, $n = 3-6$.

Table 2. Tyrosine glucoside hydrolase activity in fat body tissue from isolated abdomens of *Manduca sexta* injected with 20-hydroxyecdysone or solvent only*

| Treatment | Enzyme activity (nmol tyrosine glucoside hydrolyzed/mg protein/min \pm SEM†) |
|--------------------|---|
| 20-Hydroxyecdysone | 0.90 ± 0.30 |
| Control—solvent | 0.06 ± 0.05 |

*Larvae were ligated and injected, and the enzyme activity determined as described in Methods. †SEM = standard error of means, $n = 3$.

However, in the abdomens receiving injections of 20-hydroxyecdysone, titres of both tyrosine and tyrosine glucoside showed large changes on the third day after injection (Fig. 2B). There was a greater than 2-fold decrease in the level of tyrosine glucoside together with an increase in the titre of tyrosine. Tyrosine glucoside remained low for two days thereafter while tyrosine fell to normal levels. Pupal apolysis also occurred and the underlying new cuticle appeared to tan normally. These results demonstrated that 20-hydroxyecdysone initiates the release of tyrosine from tyrosine glucoside, perhaps by inducing or activating tyrosine glucoside hydrolase, and that it probably stimulates turnover of tyrosine itself.

Effects of 20-hydroxyecdysone on tyrosine glucoside hydrolase activity

To determine directly if 20-hydroxyecdysone is regulating the activity of the tyrosine glucoside hydrolase system, fat body was collected from isolated abdomens that had been injected with 20-hydroxyecdysone or solvent only as previously described. The abdomens receiving 20-hydroxyecdysone underwent apolysis and pupal cuticle formation as observed in the previous experiment. Fat body was removed for enzyme assays three days after injection and the start of cuticle tanning, but before ecdysis. Tyrosine glucoside hydrolase activity was fifteen times higher in the fat body exposed to the hormone than in the controls (Table 2). The observed rate of enzyme activity (0.9 nmol hydrolyzed/mg protein/min) from the ecdysone-injected abdomens would fall within the range observed in late pharate pupae between the brown metathoracic bar stage and ecdysis (Table 2). During this time span in the live animals, the hydrolase increased from 0.6 to 2.0 nmol/mg protein/min (Fig. 1). The activity in the control abdomens correlated well with the fat body enzyme levels in feeding larvae close to the wandering stage. Therefore we conclude that 20-hydroxyecdysone is regulating the activity of the tyrosine glucoside hydrolase system in the fat body of pharate pupae.

DISCUSSION

Tyrosine glucoside accumulated primarily in the haemolymph during feeding and wandering of the last larval stadium of *M. sexta*, reaching maximum concentration in the late wandering and pharate pupal stages (Ahmed *et al.*, 1983). Shortly before pupal

ecdysis, its concentration in whole body and haemolymph declined rapidly with a corresponding increase in the free tyrosine pool. The timing of the onset of tyrosine glucoside hydrolysis a few hours before pupal ecdysis suggested possible regulation of the enzyme by 20-hydroxyecdysone, which increased to high titres during this period (Bollenbacher *et al.*, 1981). Pupation is preceded by two releases of ecdysone. The first release occurs on the fourth day after ecdysis of fifth stadium larvae and triggers the cessation of feeding, purging of the gut contents and initiation of wandering behaviour. A second release occurs two days later and initiates apolysis as well as the production of pupal cuticle.

To determine if 20-hydroxyecdysone, which initiates pupal cuticle formation in *M. sexta*, also induces or activates the hydrolase, isolated larval abdomens were injected with the hormone to mimic its titres in the haemolymph (Nijhout, 1976). The decrease in tyrosine glucoside and the corresponding rise in free tyrosine in the haemolymph of the ecdysone injected abdomens demonstrated that hormone titres large enough to induce cuticle formation and ecdysis also stimulates cleavage of the glucoside. We further verified that tyrosine glucoside hydrolase activity in fat body was increased about fifteen times above control levels in response to the hormone. Whether 20-hydroxyecdysone has a primary or secondary effect on the induction or activation of the hydrolase is unknown. The absence of an immediate response to moulting hormone suggests that a secondary regulator is responsible for controlling hydrolase activity.

The increase in tyrosine glucoside hydrolase activity in response to high titres of 20-hydroxyecdysone may be a regulatory step in the sequence of events leading to pupal cuticle sclerotization. Previously Bodnaryk (1970b, 1971) had shown that ecdysone mediated the hydrolysis of phenylalanine and tyrosine storage peptides in *M. domestica* and *S. bullata*, respectively. The large pool of free tyrosine required for sclerotization of the puparium of Diptera and pupal cuticle of Lepidoptera is therefore supplied at the precise time by the same hormonal command that initiates other metamorphic events related to cuticle formation and ecdysis.

Our results also showed that fat body of newly ecdysed pupae contained the highest level of tyrosine glucoside hydrolase activity. Hydrolase activity was very low in the fat body of feeding fifth stadium larvae, but increased approx five times in the wandering and pharate pupal stages. Dissected fat body from newly ecdysed pupae would serve as an excellent source of the glucosidase enzyme for its purification and characterization.

The first small release of ecdysone that initiates wandering behaviour may be responsible for the first increase in hydrolase activity which catalyses the slow build up of free tyrosine in the late pharate pupa (Ahmed *et al.*, 1983). The second release of ecdysone probably leads to the maximum levels of β -glucosidase at the time of pupal ecdysis when tyrosine is most rapidly metabolized to catecholamines (Hopkins *et al.*, 1982). A second source of the free tyrosine pool may be the hornworm storage protein "manducin." This is the predominant hae-

molymph protein in the fifth larval stage and contains relatively large amounts of tyrosine and phenylalanine residues (Kramer *et al.*, 1980b). Manducin reaches peak titres toward the end of larval feeding and begins to decline during the wandering and pharate pupal stages. The regulation of amino acid release from manducin is unknown.

The localization of tyrosine glucoside hydrolase in fat body points to the importance of transport systems for moving tyrosine glucoside, tyrosine and other metabolites between tissues of synthesis, storage, release and utilization. The haemolymph acts as the major reservoir for both tyrosine and tyrosine glucoside prior to cuticle tanning. Tyrosine glucoside must then be transported into fat body cells, the site of the β -glucosidase, for cleaving the conjugate. Free tyrosine diffuses back into the haemolymph where it is metabolized primarily to *N*- β -alanyldopamine for pupal cuticle tanning (Hopkins *et al.*, 1982). The regulation of tyrosine storage, transport and metabolism to sclerotizing agents in precise synchrony with other developmental events is of critical importance to new exoskeleton formation by insects.

REFERENCES

- Adams R. F., Vandemark F. L. and Schmidt G. J. (1977) Ultramicro GC determination of amino acids using glass open tubular columns and a nitrogen-selective detector. *J. Chromatog. Sci.* **15**, 63-68.
- Ahmed R. F., Hopkins T. L. and Kramer K. J. (1983) Tyrosine and tyrosine glucoside titres in whole animals and tissues during development of the tobacco hornworm *Manduca sexta* (L.). *Insect Biochem.* **13**, 369-374.
- Bell R. A. and Joachim F. G. (1976) Techniques for rearing laboratory colonies of tobacco hornworm and pink bollworms. *A. Ent. Soc. Am.* **69**, 365-373.
- Bodnaryk R. P. (1970a) Levels of free glutamic acid, phenylalanine and γ -glutamyl-L-phenylalanine during pupal sclerotization in the house fly, *Musca domestica* L. *Comp. Biochem. Physiol.* **35**, 499-502.
- Bodnaryk R. P. (1970b) Biosynthesis of γ -L-glutamyl-L-phenylalanine by the larva of the housefly *Musca domestica*. *J. Insect. Physiol.* **16**, 919-929.
- Bodnaryk R. P. (1971) Effect of exogenous molting hormone (ecdysterone) on β -alanyl-L-tyrosine metabolism in the larva of the fly *Sarcophaga bullata* Parker. *Gen. Comp. Endocr.* **16**, 363-368.
- Bodnaryk R. P. (1972) A survey of the occurrence of β -alanyl-tyrosine, γ -glutamyl-phenylalanine and tyrosine-O-phosphate in the larval stage of flies (Diptera). *Comp. Biochem. Physiol.* **43B**, 587-592.
- Bollenbacher W. E., Smith S. L., Goodman W. and Gilbert L. I. (1981) Ecdysteroid titer during larval-pupal-adult development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocr.* **44**, 302-306.
- Bradford M. M. (1976) A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72**, 248-254.
- Brunet P. C. J. (1980) The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. *Insect Biochem.* **10**, 467-500.
- Chen P. S., Mitchell H. K. and Neuweg M. (1978) Tyrosine glucoside in *Drosophila busckii*. *Insect Biochem.* **8**, 279-286.
- Dunn P. E., Fader R. G. and Regnier F. E. (1977) Metabolism of β -alanyl-L-tyrosine in *Sarcophaga bullata*. *J. Insect Physiol.* **23**, 1021-1029.
- Hopkins T. L., Morgan T. D., Aso Y. and Kramer K. J.

- (1982) *N*- β -Alanyldopamine: major role in insect cuticle tanning. *Science* **217**, 364-366.
- Ishizaki Y. and Umebachi Y. (1980) The presence of tyrosine glucoside in the haemolymph of lepidopteran insects. *Scient. Rep. Kanazawa Univ.* **25**, 43-52.
- Isobe M., Kondo N., Imai K., Yamashita O. and Goto T. (1981) Glucosyltyrosine in silkworm haemolymph haemolymph as a transient metabolite of insects. *Agric. Biol. Chem.* **45**, 687-692.
- Kramer K. J., Hopkins T. L., Ahmed R. F., Mueller D. and Lookhart G. (1980a) Tyrosine metabolism for cuticle tanning in the tobacco hornworm, *Manduca sexta* (L.) and other Lepidoptera: identification of β -D-glucopyranosyl-*O*L-tyrosine and other metabolites. *Archs Biochem. Biophys.* **205**, 146-155.
- Kramer S. J., Mundall E. C. and Law J. H. (1980b) Purification and properties of manducin, an amino acid storage protein of the haemolymph of larval and pupal *Manduca sexta*. *Insect Biochem.* **10**, 279-288.
- Levenbook L., Bodnaryk R. P. and Spande T. F. (1969) β -Alanyl-L-tyrosine, chemical synthesis, properties and occurrence in larvae of the fleshfly *Sarcophaga bullata* Parker. *Biochem. J.* **113**, 837-841.
- Lu P., Kramer K. J., Seib P. A., Mueller D. D., Ahmed R. and Hopkins T. L. (1982) β -D-Glucopyranosyl-*O*-L-tyrosine: synthesis, properties and titre during insect development. *Insect Biochem.* **12**, 377-381.
- Lunan K. D. and Mitchell H. K. (1969) The metabolism of tyrosine-*O*-phosphate in *Drosophila*. *Archs Biochem. Biophys.* **132**, 450-456.
- Mitchell H. K. and Lunan K. D. (1964) Tyrosine-*O*-phosphate in *Drosophila*. *Archs Biochem. Biophys.* **106**, 219-222.
- Nijhout H. F. (1976) The role of ecdysone in pupation of *Manduca sexta*. *J. Insect. Physiol.* **22**, 453-463.
- Wirtz R. A. and Hopkins T. L. (1977) Tyrosine and phenylalanine concentrations in the cockroaches *Leucophaea maderae* (F.) and *Periplaneta americana* (L.) in relation to cuticle formation and ecdysis. *Comp. Biochem. Physiol.* **56A**, 263-266.