

## ROLE OF JUVENILE HORMONE IN REGULATING TYROSINE GLUCOSIDE SYNTHESIS FOR PUPAL TANNING IN *MANDUCA SEXTA* (L.)

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**Abstract**—Tyrosine glucoside ( $\beta$ -D-glucopyranosyl-O-L-tyrosine) serves as a reservoir of tyrosine and glucose for pupal and adult cuticle formation, tanning, and pigmentation in several Lepidopteran insects. In the tobacco hornworm, *Manduca sexta* (L.), detectable quantities appear in the haemolymph 1–2 days after ecdysis of the fifth instar and very high concentrations accumulate between the fourth and eighth days of the stadium. If juvenile hormone II or a mimic (methoprene) is injected into fifth-instar larvae at 24-h intervals after ecdysis, tyrosine glucoside synthesis is almost completely suppressed. Temporary starvation of newly ecdysed larvae that results in the maintenance of a high endogenous juvenile hormone titre, also suppresses and delays the onset of tyrosine glucoside synthesis. The decrease and eventual disappearance of juvenile hormone after ecdysis of the last-larval instar appears to be a necessary prerequisite for the synthesis or activation of tyrosine glucoside synthetase along with the initiation of other metamorphic events.

**Key Word Index:** Juvenile hormone, tyrosine, tyrosine glucoside, cuticle, tanning, *Manduca sexta*, tobacco hornworm, tyrosine glucoside synthesis

### INTRODUCTION

Tyrosine glucoside ( $\beta$ -D-glucopyranosyl-O-L-tyrosine) serves as a readily mobilizable source of tyrosine and glucose in the haemolymph of many Lepidoptera (Ishizaki and Umebachi, 1980; Kramer *et al.*, 1980; Isobe *et al.*, 1981; Lu *et al.*, 1982) and in *Drosophila busckii* (Chen *et al.*, 1978). Based on its developmental profiles, this storage conjugate is associated mainly with pupal and adult cuticle sclerotization and pigmentation. In *Manduca sexta* tyrosine glucoside first appears in the haemolymph 24–48 h after ecdysis of the fifth-larval instar and increases rapidly as feeding ceases and wandering behaviour begins (Ahmed *et al.*, 1983a). The onset of tyrosine glucoside synthesis early in the last-larval stadium suggested a possible hormonal mechanism for regulation of the synthetase system in conjunction with initiation of other metamorphic events. Juvenile hormone titre is very high in ecdysing fifth-instar larvae but drops about 6-fold and then remains at an intermediate concentration through the next 2 days (Fain and Riddiford, 1975). Juvenile hormone titre rapidly declines thereafter to non-detectable levels in mature larvae (Fain and Riddiford, 1975; Nijhout and Williams, 1974). The absence of tyrosine glucoside throughout most of larval development and its first appearance and subsequent large accumulation in the last instar prior to metamorphosis suggested an inverse correlation with the pattern of juvenile hormone titre.

To determine if the clearance of juvenile hormone from the last-larval instar is a necessary prerequisite for the synthesis of tyrosine glucoside, we have investigated the effect of exogenous juvenile hormone II and a mimic (methoprene) treatment on the normal pattern of accumulation of this conjugate. We have also used temporary starvation of newly ecdysed larvae to study the effect of maintaining a high endogenous juvenile hormone titre on tyrosine glucoside and tyrosine concentrations in the haemolymph. Starvation of newly ecdysed fifth-instar larvae of *M. sexta* has been shown to result in an actual increase in juvenile hormone titre beyond the point where it normally decreases and disappears (Nijhout, 1975; Cymborowski *et al.*, 1982). The hormonal regulation of tyrosine glucoside synthesis and hydrolysis is discussed in relation to the switch from larval development to metamorphosis.

### MATERIALS AND METHODS

#### *Experimental animals*

*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 photoregime of 16 h light:8 h dark.

#### *Chemicals*

Juvenile hormone II [(2E,6E)-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoic acid methyl ester] was obtained from Sigma Chemical Co. The juvenile hormone mimic, methoprene [isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate] was a gift from Zocon Corporation (Palo Alto, CA). Stock solutions of the hormonal compounds were made up

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in hexane and then aliquots were evaporated under nitrogen and dissolved in olive oil ( $2.5 \mu\text{g}/\mu\text{l}$ ) for injection into larvae.

#### Hormone injection experiments

Fifth-stadium larvae were injected with solutions of juvenile hormone II or methoprene starting 1 day after ecdysis and at daily intervals until 24 h before haemolymph was collected for analysis of tyrosine and tyrosine glucoside. After immobilization on ice, the animals were injected with a microsyringe through an abdominal proleg. Each animal was dosed with juvenile hormone II or methoprene at the rate of  $5 \mu\text{g}/\text{ml}$  haemolymph based on the haemolymph volume representing 40% of larval body weight (William-Boyce and Jungreis, 1980). The controls received olive oil only ( $2 \mu\text{l}/\text{ml}$  haemolymph). Haemolymph was individually collected from three larvae at each time interval for analysis of tyrosine and tyrosine glucoside (Ahmed *et al.*, 1983a).

#### Starvation experiments

Fifth-stadium larvae were starved for the initial 72 h following ecdysis and then placed on artificial diet (Jones *et al.*, 1980). Haemolymph was collected individually from three larvae at 24-h intervals beginning at 48 h after ecdysis for analysis of tyrosine and tyrosine glucoside concentrations.

#### Tyrosine and tyrosine glucoside analysis

Haemolymph was extracted in cold 80% methanol containing 5 mM ascorbate. After centrifugation at 12,000 g for 15 min at  $4^\circ\text{C}$ , 0.25 ml aliquots of the supernatant were acidified with 0.75 ml 25% acetic acid to pH 3 and cleaned by passage through a small column containing 200 mg of DOWEX 50-W-X2 200-400 mesh, hydrogen form (Bio-Rad Co.). The column was washed with 1 ml deionized water and the amino acids eluted with 1 ml 2 M ammonium hydroxide. Aliquots were dried and analyzed for tyrosine and tyrosine glucoside by gas-liquid chromatography as described by Ahmed *et al.* (1983a).

## RESULTS

#### Effect of injection of juvenile hormone and methoprene

The control larvae receiving injections of olive oil solvent every 24 h had haemolymph concentrations of tyrosine and tyrosine glucoside very similar to those previously observed in normal fifth-stadium larvae (Ahmed *et al.*, 1983a). Tyrosine glucoside increased sharply from 0.3 to 3.6 mg/ml of haemolymph, between 90 and 130 h after ecdysis of the last instar until the last observation at 134 h (Fig. 1A). Tyrosine also showed a gradual increase from 0.6 to 0.8 mg/ml haemolymph over the same interval.

When larvae were injected with either juvenile hormone II or methoprene in olive oil at 24-h intervals after ecdysis, tyrosine glucoside was detected only at very low concentrations, less than 0.1 mg/ml of haemolymph (Fig. 1B and C) even though these animals gained weight at a rate comparable to the control animals. Free tyrosine was present in higher concentrations than tyrosine glucoside but also at substantially lower concentrations than in the control animals. Unlike the tyrosine concentrations in con-

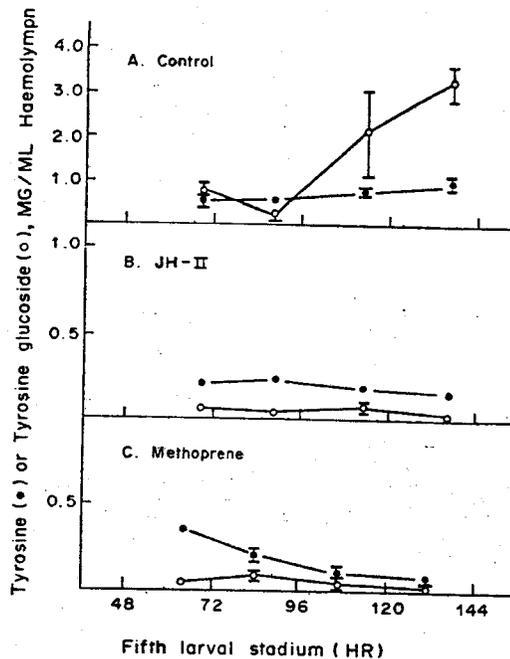


Fig. 1. Effect of juvenile hormone (JH II) and methoprene on tyrosine and tyrosine glucoside concentrations in the haemolymph of fifth-instar *M. sexta* larvae. Injections were made at 24-h intervals starting 24 h after ecdysis. Controls received solvent (olive oil) only, with wandering behaviour occurring at approx 120 h. Vertical bars are  $\pm$ SEM,  $n = 3$ .

trol animals, tyrosine exhibited no tendency to increase when juvenile hormone or the mimic was injected. Four to five days after injections of hormone ceased, the larvae began wandering behaviour indicating that the hormone had been inactivated or cleared from the haemolymph, and that it had had only a transitory effect in delaying metamorphosis. However, methoprene-injected fifth-instar larvae did not undergo metamorphosis after treatment was stopped, but instead they moulted about 10 days after injections ceased to sixth instar larvae which died during ecdysis.

#### Effect of starvation

Fifth-stadium larvae were starved for 72 h after ecdysis and then provided with artificial diet in order to extend the period of development when endogenous titre of juvenile hormone is high. Although tyrosine glucoside normally begins to increase in haemolymph by 48 h after ecdysis of the fifth instar, no increase was observed in starved larvae until 144 h (72 h after being placed back on diet) [Fig. 2]. A very large increase in tyrosine glucoside occurred by 168 h to a level similar in concentration to that present in normally fed 144-h larvae. Free tyrosine showed a gradual increase in concentration after the larvae were supplied with diet, reaching near normal levels after 2 days of feeding. Therefore, one of the effects of temporary starvation and subsequent elevated juvenile hormone titre was to delay the onset of tyrosine glucoside synthesis.

In regard to morphological effects, a total of 33 larvae similarly starved were held for observation. Fourteen larvae (42%) that were starved for 3 days

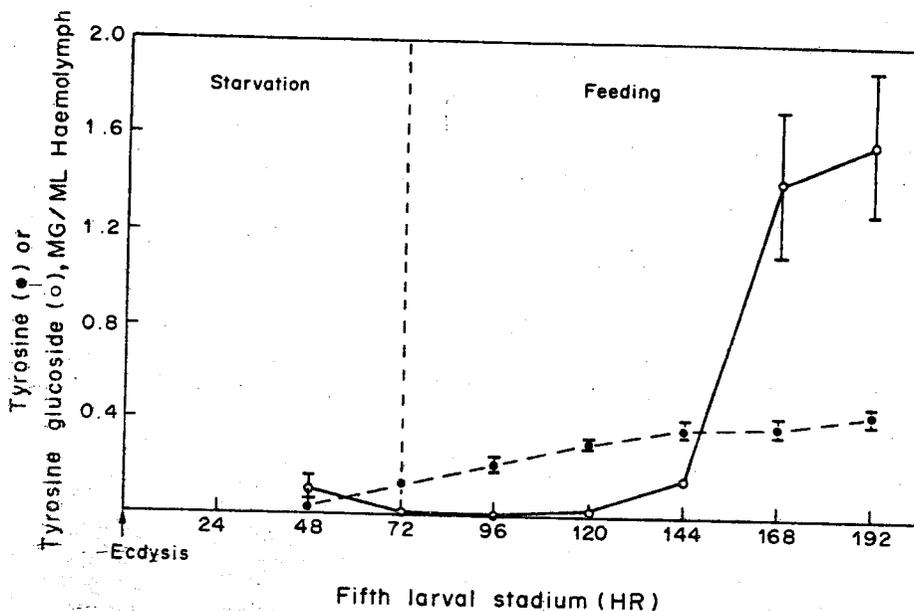


Fig. 2. Tyrosine and tyrosine glucoside concentrations in haemolymph of fifth-instar *M. sexta* larvae starved for 72 h and then fed artificial diet. Vertical bars are  $\pm$  SEM,  $n = 3$ .

wandered after an average of 10.5 days, pupated after an average of 17 days and all eclosed as adults. Ten larvae (30%) underwent a supernumerary moult to a sixth instar but all died without feeding. The mouthparts of these oversized larvae were malformed. Nine larvae (28%) died from unknown causes before undergoing pupation.

#### DISCUSSION

The hypothesis that the tyrosine glucoside synthetase system is regulated by a declining titre of juvenile hormone in haemolymph was supported by data obtained from experiments where either juvenile hormone II or methoprene was injected into fifth-stage larvae at 24-h intervals after ecdysis to maintain a high level of hormonal activity. At this time, endogenous juvenile hormone titres would normally have fallen to intermediate levels, allowing synthesis or activation of the synthetase enzyme. However, sustained treatment with either exogenous hormone or its mimic essentially prevented production of the conjugate. Tyrosine concentrations also remain abnormally low compared to the controls throughout the treatment period. Since free tyrosine cannot be sequestered as tyrosine glucoside in the hormone-treated animals, one might expect its concentration to increase due to digestion of dietary protein and absorption into the haemolymph. Just the opposite was observed indicating that high juvenile hormone titres may have multiple effects on tyrosine metabolism.

Extended maintenance of high endogenous juvenile hormone titres in last instar larvae of *M. sexta* is possible by starvation of newly ecdysed larvae for variable lengths of time (Nijhout and Williams, 1974; Nijhout, 1975; Cymborowski *et al.*, 1982). Jones *et al.* (1980) found that the optimal condition for maximal

survival and supernumerary moulting was 3 days of starvation with only agar medium provided as a water source. Cymborowski *et al.* (1982) reported that 5 days of starvation was necessary for a high percentage of supernumerary moulting even though the titre of juvenile hormone remained high and even increased through the first 4 days of starvation. When we starved newly ecdysed fifth-instar larvae for 72 h, tyrosine glucoside synthesis was completely suppressed through 120 h after ecdysis. A small increase in haemolymph concentration of the conjugate occurred at 144 h and a very large increase to 1.4 mg/ml by 168 h or 4 days after the larvae had resumed feeding. Haemolymph concentrations of tyrosine showed a gradual increase after resumption of feeding to a level of 0.4 mg/ml by 168 h. From a similar group of larvae observed for the effects of starvation, more than half of the survivors pupated and eclosed as adults. About 40% of the survivors moulted to sixth instar larvae, a result somewhat intermediate between the supernumerary moulting percentages of Jones *et al.*, (1980) and Cymborowski *et al.* (1982).

Therefore, the effect of starvation in elevating endogenous juvenile hormone titres and prolonging larval development also temporarily delays the onset of tyrosine glucoside synthesis. It would appear that the genetic programme for synthesis or activation of the enzyme system for production of tyrosine glucoside during the last-larval stadium cannot be expressed until the titre of juvenile hormone normally declines and ultimately disappears from the haemolymph in concert with other metamorphic events. Since tyrosine glucoside is not detected in the penultimate-larval stage, we postulate that the high juvenile hormone titre that occurs during the first 2 days after ecdysis to the fourth larval instar prevents expression of this enzyme activity. Fain and Riddiford (1975) have shown that juvenile hormone titres



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