EXCRETORY ROLE OF THE MIDGUT IN LARVAE OF THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)*

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SUMMARY

Caterpillars of *Manduca sexta* use two distinct transport mechanisms for the excretion of dyes. One pump (Type A) has a high affinity for acid (anionic) dyes and occurs in the midgut and medial Malpighian tubules. Acid dyes accumulate rapidly in the lumen of the midgut while the Malpighian tubules appear to play only a minor role in the excretion of these dyes. The other pump (Type B) excretes basic (cationic) dyes and is located primarily in the proximal Malpighian tubules. Evidence is presented that hippuric acid competes with acid dyes for excretion by both midgut and Malpighian tubules. After the final-instar larva purges its gut the ability of the midgut and Malpighian tubules to excrete dyes gradually decreases. Sixty hours after the purge only the Malpighian tubules retain some dye excreting activity.

INTRODUCTION

One of the important functions of excretory systems is the elimination of harmful substances from the body fluids. The injection of dyes such as indigo carmine and phenol red has been classically used to demonstrate if and where such excretion occurs. This method of 'physiological injection' has been an invaluable tool in elucidating the mechanisms of excretion in vertebrates and in many invertebrates. Extensive studies by Lison (1937, 1938) and Palm (1952) established that in insects dyes are excreted almost exclusively by the Malpighian tubules. In addition the pericardial cells, nephrocytes and labial glands may become involved in dye excretion depending on the chemical nature of the dye, the concentration in which it is injected and the species of insect involved (Palm, 1952).

Several investigators have observed the appearance of injected dyes in the lumen of the midgut of various insects (Grassé & Lesperon, 1935; Lesperon, 1937; Gersch, 1942). Although Palm (1952) verified the observations of these authors he found that the excretory ability of the midgut becomes evident only when particular concentrations of dyes are used, and he did not consider the midgut as playing a significant role in excretion.

I have found that larvae of the tobacco hornworm, *Manduca sexta*, appear to be unique in that certain dyes are excreted almost exclusively by the midgut. During

* Lepidoptera: Sphingidae.
a series of pilot experiments dyes were injected in a large range of concentrations and in each case the midgut was clearly the major organ of excretion. This paper examines the physiological properties and relative roles of the Malpighian tubules and midgut of Manduca in the excretion of dyes.

MATERIALS AND METHODS

Larvae of Manduca sexta were reared as described by Truman (1972). All individuals used in these experiments were final (5th) instar larvae weighing 7-9 g, unless stated otherwise. All experiments were performed at room temperature which ranged from 22 to 24 °C.

Dyes were used as $2 \times 10^{-2} \text{M}$ solutions (except where noted) in a saline modified from that of Weevers (1966) by Cherbas (1973), buffered at pH 6-8. Those dyes which were not soluble at this concentration were used as saturated solutions. All dyes used in this study were certified by the Biological Stain Commission.

Prior to injection of the dye the larvae were deeply anaesthetized with CO$_2$. Depending on the colour intensity, 100 or 150 μl of the dye solution were injected into the base of a proleg.

To examine the progress of dye excretion, individuals were dissected at 5 min intervals after injection for the first 30-45 min. In cases where excretion was very slow, dissections were done at hourly intervals up to 6 h after injection. Excretion of dye was determined visually by dissecting larvae and examining the contents of the Malpighian tubules and midgut for presence of dye. Basic dyes caused an often intense staining of the cytoplasm of the Malpighian tubules. It was therefore necessary to puncture the tubules to ascertain whether dye was present in the lumen.

The rate of amaranth excretion by isolated pieces of midgut was determined by a procedure similar to that used by Crane & Wilson (1958) to study the rate of sugar transport across the rat intestine. A piece of midgut 2-3 cm in length was carefully removed from a larva and slipped over a length of polyethylene tubing which was flared at one end. One end of the gut was snugly ligated to the tube, just before the flare. The free end was then carefully rolled over the ligated end until the gut was completely everted. The free end was then ligated tightly with cotton thread (Fig. 1A). The resulting bag was filled with 100-200 μl of a $2 \times 10^{-2} \text{M}$ dye solution with the aid of a long hypodermic needle attached to a syringe. About half of the injected solution usually backed up into the polyethylene tube which thus acted as a reservoir. This preparation was suspended in a Plexiglass cuvette containing 4 ml of saline, mounted in a Zeiss spectrophotometer. A gentle stream of oxygen was bubbled through the saline and served to oxygenate and mix the bathing medium. The path of the light beam was positioned in such a way that no interference from the gut bag or bubbles resulted (Fig. 1B). Readings were made at 522.5 nm, the absorption maximum of amaranth. A continuous record of the optical density (o.d.) of the medium was kept by means of a Corning pen recorder which plotted o.d. as a linear function of time. The resulting curve was normally linear from about 3 up to 90 min after initiation of the experiment. The slope of the curve for the first half hour of the experiment was measured and used as a basis for determining the rate of dye excretion. At the end of the experiment the gut bag was tested for leaks by applying a low pressure to the
liquid in the polyethylene tube by means of a syringe. If leakage was suspected the experiment was discarded. Occasionally a leak became apparent through a sudden rise in the rate of appearance of the dye midway through the experiment.

RESULTS

(1) Anatomy of the excretory system

The arrangement of the Malpighian tubules in larvae of *Manduca sexta* is typical of Lepidoptera. There are 6 tubules arranged in two groups which meet in a common vesicle at their base before entering the anterior part of the hindgut at the level of the 7th abdominal segment (Fig. 2A). Morphologically and physiologically each tubule is differentiated into 3 parts. (1) The proximal tubule runs anteriorly along the midgut from the common vesicle to the 2nd or 3rd abdominal segment. This part has a relatively smooth surface with shallow diverticula (Fig. 2B). (2) The medial tubule runs posteriorly along the midgut parallel to the proximal tubule, to the 6th abdominal segment where it becomes highly folded. This part is studded with knoblike diverticula (Fig. 2C). (3) The distal tubule lies entirely in the 6th and 7th abdominal segments and is highly convoluted. It resembles the medial tubule in morphology (Fig. 2D), but is yellow in colour. Posteriorly it is completely transparent and exceedingly delicate. Its distal tip invades the wall of the hindgut.

At the outset of the instar the entire Malpighian tubule is clear and transparent. Approximately 24 h after the moult to the final instar, when the larvae weigh about 2.5 g, a white precipitate begins to appear in the tubules. The distal and medial parts of the tubules of larvae weighing 5 g or more are replete with this substance (presumably uric acid) which flows out readily when the tubules are punctured. This precipitate also appears in the anterior part of the distal tubules. As will be described in the next section, only the proximal and medial parts of the tubules participate in dye excretion.
Fig. 2. Lateral view of the excretory system of a *Manduca* larva. A, arrangement of the Malpighian tubules. The distal part is shown for the dorsal tubule only. B, detail of the proximal part of the tubule. C, detail of the medial part. D, detail of the distal part. The position of the abdominal segments (*A*$_5$-*A$_7$) is indicated on the scale below. *d*, dorsal tubule; *l*, lateral tubule; *v*, ventral tubule; *vs*, common vesicle; *m*, midgut; *di*, diverticulum.

(2) The excretion of water-soluble dyes

The pathway of excretion of 24 water-soluble dyes was investigated. Table 1 shows that these dyes can be subdivided into 3 categories. (a) Those that are excreted by the midgut and the medial Malpighian tubules. (b) Those that are excreted primarily by the proximal Malpighian tubules. (c) Those that are not visibly excreted over a 6 h period. All dyes in category (a) are acid dyes, whereas those in categories (b) and (c) are basic dyes.

In order to ensure that the dyes in category (a) did not enter the midgut via the Malpighian tubules, the following experiment was performed. A blood-tight ligature was applied between the 5th and 6th abdominal segment, well anterior to the junction of the Malpighian tubules and hindgut. Dye was then injected into the anterior section of the ligated larva. The animals were dissected after 30 and 60 min. Presence of dye in the lumen of the midgut was taken as evidence for intrinsic excretory activity of this organ. Excretion by the midgut was verified in this manner for all dyes in category (a). Not all dyes were excreted at the same rate. Among those in category (a), brom cresol purple, chlor phenol red, fast green and indigo sulphonate were detected in the lumen of the midgut within 5–10 min of injection, whereas the remaining dyes in this category did not become evident in the gut until 20–30 min after injection.

In category (b), methylene blue and methyl green were excreted particularly rapidly. These dyes appeared simultaneously in both the proximal and medial tubules. Furthermore, methyl green was also excreted at a very low rate by the midgut where traces of this dye were detectable one hour after injection. These observations suggested that the transport mechanisms for dyes in categories (a) and (b) were not restricted to a particular area of the Malpighian tubules or midgut. They probably occur throughout the excretory system with a higher concentration in some areas than in others. If this
Excretion by midgut in Manduca

Table 1. Sites of excretion of water-soluble dyes by larvae of Manduca sexta

<table>
<thead>
<tr>
<th>Organ of excretion*</th>
<th>Malpighian tubule</th>
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<tbody>
<tr>
<td>Dye</td>
<td>Proximal†</td>
<td>Medial</td>
<td>Midgut</td>
<td></td>
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<tr>
<td>Category a</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Brom cresol purple</td>
<td>–</td>
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<td>Chlor phenol red</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>Fast green</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Indigo sulphonate</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Buffalo black NBR</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Fluorescein (sodium)</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Chlorazol black E</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Acid fuchsin</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Amaranth</td>
<td>–</td>
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<td>Ponceau S</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Chromotrope 2R</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>Category b</td>
<td></td>
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<tr>
<td>Methyl green</td>
<td>++</td>
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<tr>
<td>Methylene blue</td>
<td>+++</td>
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<tr>
<td>Azure B</td>
<td>++</td>
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<tr>
<td>Toluidine blue</td>
<td>+</td>
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<tr>
<td>Neutral red</td>
<td>++</td>
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<tr>
<td>Pyronin Y</td>
<td>++</td>
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<tr>
<td>Basic fuchsin</td>
<td>+</td>
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<tr>
<td>Category c</td>
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<tr>
<td>Gentian (crystal) violet</td>
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<td>Malachite green</td>
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<tr>
<td>Phloxine B</td>
<td>–</td>
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<tr>
<td>Ethyl violet</td>
<td>–</td>
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<tr>
<td>Alcian blue</td>
<td>–</td>
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<tr>
<td>Coomassie (brilliant) blue</td>
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* Number of symbols indicates relative rate of excretion by each organ.
† Excretion of dyes in category a by the proximal tubule cannot always be ascertained as dye excreted by the medial tubule soon fills it up.

is indeed the case, then any dye in category (b) could be excreted by the midgut if enough time was allowed. This proposition was tested by injecting 50 μl of a saturated methylene blue solution into larvae previously ligated between the 5th and 6th abdominal segments. These larvae were dissected 24 and 36 h after the injection and were found to have substantial amounts of methylene blue in the lumen of the midgut.

(3) Rate of amaranth excretion by the midgut, in vitro

Amaranth, like all other acid dyes tested, is excreted primarily by the midgut and to a lesser extent by the distal Malpighian tubules. Substantial excretion of dyes by the midgut has not been demonstrated previously, hence I have made an attempt to determine the rate of excretion of this dye by isolated pieces of midgut, as described under Methods. Fig. 3 shows a representative trace of the time course of dye excretion. The mean rate of excretion was $1.12 \pm 0.36 \times 10^{-6} M/h/mg$ (dry weight of midgut). The portion of the midgut over which excretion occurs in a larva (weighing between 7–9 g) is approximately 4.5 cm long and has a dry weight of 19.7 mg/cm. The mean rate of excretion for an entire midgut is therefore about $9.9 \times 10^{-6} M/h$. 

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Inhibition by 2,4-dinitrophenol (DNP)

To test the effect of DNP (Na salt) on the dye excreting mechanism, larvae were injected with 100 μl of an isotonic saline solution containing 8 × 10⁻² M-DNP and 4 × 10⁻² M dye. The dyes used in this experiment were amaranth and methylene blue. Control larvae were injected with 100 μl of 4 × 10⁻² M dye in saline. Larvae that received DNP were massaged gently every 10–15 min to aid their circulation. Larvae were dissected 1 h after injection. Almost all the injected methylene blue and amaranth was found in the Malpighian tubules and midgut, respectively, of the control larvae. By contrast, only traces of the dyes were excreted in larvae that also received DNP.

Inhibition by DNP was also tested on isolated Malpighian tubules in an incubation medium consisting of isotonic saline containing 10⁻³ M-DNP and 6 × 10⁻⁴ M dye. The dyes used in this experiment were neutral red, methylene blue and chlor phenol red. When necessary the pH of the medium was adjusted to 6-8. Proximal and medial segments of the Malpighian tubules were incubated in this medium for up to 45 min. Tubules incubated in a 6 × 10⁻⁴ M solution of dye in saline served as controls. The tubules were removed to clean saline at 5 min intervals for examination and comparison with controls. They were always ripped open to determine the presence of dye in the lumen. Tubules incubated in a DNP containing medium took up little or no dye while controls showed a considerable uptake of the dye.

Competition with hippuric acid

In vertebrates and invertebrates benzoic acid and some of its higher homologues are converted to hippuric acid by conjugation with glycine (H. Smith, 1951; J. Smith, 1955). The conversion to hippuric acid is evidently a prerequisite for the effective excretion of these toxic substances which occur in abundance in the diet of herbivores. Smith & Ranges (1938) have shown that, in man, high titres of hippuric acid decrease
the rate of phenol red clearance by the kidney tubules through competition for common transport sites. This observation suggests that selective excretion of dyes by mammals results from the fact that they have a structural resemblance to toxic aromatic compounds, such as hippuric acid, for which a specific excretory mechanism exists (Smith, 1951). Insects are known to synthesize and excrete hippuric acid (Friedler & Smith, 1954; Casida, 1955). It is therefore possible that, by analogy with vertebrates, the excretion of dyes in insects is mediated by the same mechanism that is normally responsible for the excretion of hippuric acid and similar toxic substances (Maddrell, 1971). If this is indeed the case then the rate of dye excretion should diminish in the presence of hippuric acid as both molecules would be competing for sites on the same transport mechanism.

(a) Competition in vivo

Amaranth and indigo sulphonate were the dyes selected to test for competition with hippuric acid. Two hundred microlitres of an isotonic saline solution containing \(10^{-2}\) M dye and \(0.1\) M sodium hippurate was injected into the haemocoel of each test larva. Control larvae were injected with 200 \(\mu l\) dye solution. Larvae were dissected 30 min after the injection and the contents of the Malpighian tubules and midgut were examined. In all instances the larvae that had received hippuric acid had markedly smaller amounts of dye in the lumen of their midgut than did control larvae. No difference was seen in the dye content of the Malpighian tubules using this technique.

(b) Competition in vitro

The effect of hippuric acid on the rate of amaranth excretion by isolated pieces of midgut was determined by the technique described under Methods. Isolated gut bags were filled with an isotonic saline solution containing \(0.1\) M sodium hippurate and \(10^{-2}\) M amaranth. \(0.1\) M-NaCl was substituted for hippurate in control experiments, in which the mean rate of amaranth excretion was \(11.8 \pm 0.25 \times 10^{-8} \text{M/h}\) \((n = 5)\). In the presence of hippuric acid this rate decreased to \(6.2 \pm 1.3 \times 10^{-8} \text{M/h}\) \((n = 5)\).

Competition of dye and hippuric acid for excretion by isolated Malpighian tubules was also examined. Of each larva one set of 3 tubules was incubated in the experimental medium while the other set was incubated in the control medium. The experimental medium was made up of 1 part isotonic saline, 1 part \(0.15\) M sodium hippurate and \(1/30\) part \(2 \times 10^{-2}\) M dye. In the control medium \(0.15\) M-NaCl was substituted for the hippurate. The pH of all media was adjusted to 6.8 when necessary. Tubules were removed from the incubation medium to clean saline at 5-10 min intervals for a period of 45-60 min. The tubules were ripped open so that the contents of the lumen of experimentals and controls could be compared. A marked inhibition of dye uptake in the presence of hippuric acid was evident for brom cresol purple, chlor phenol red and acid fuchsin and to a lesser extent for amaranth. All these dyes belong in category \(a\) (Table 1). By contrast, the presence of hippuric acid did not interfere with the uptake of basic dyes of category \(b\) such as methylene blue, neutral red or methyl green by isolated tubules. The preferred excretion of acid dyes by the medial tubules and of neutral red by the proximal tubules was clearly evident in this in vitro experiment.
Four days prior to pupation, larvae of *Manduca sexta* cease to feed and undergo a massive purging of the gut contents. The animal will not resume feeding for about 3 weeks, the time required for adult development. It is reasonable to assume that in the absence of food intake there will be an accompanying change in the requirements imposed on the excretory system.

I therefore examined the ability of larvae to excrete chlor phenol red and methylene blue at various times after the gut purge. Larvae were injected with 100 μl of a \(2 \times 10^{-2}\) M solution of these dyes. They were dissected 1 h after the injection and the contents of the Malpighian tubules and midgut were examined for the presence of dye. These observations revealed that at 12 h after the gut purge both dyes were rapidly excreted, by the midgut and Malpighian tubules, respectively. By 24 h after the purge there was a marked decrease in the excretion of chlor phenol red by the midgut, and by 48 h after the purge no dye was excreted into the lumen of the gut within the period of observation. A similar progressive decrease in dye excretion was evident in the Malpighian tubules. By 36 h after the purge the rate of methylene blue and chlor phenol red excretion had decreased considerably, and at 60 h only traces of the dyes appeared in the tubules after a 1 h exposure. Pupation occurs about 4 days after the gut purge and the remodelling of the larval digestive and excretory system into that of the adult begins shortly thereafter.

**DISCUSSION**

Larvae of *Manduca sexta* appear to use two types of pumps for the excretion of dyes. One (Type A) has a high affinity for acid (anionic) dyes and is located principally in the midgut and medial Malpighian tubules. The other (Type B) excretes basic (cationic) dyes. It occurs in the Malpighian tubules and appears to attain its highest activity in the proximal parts of the tubules. The fact that methyl green and methylene blue are excreted very slowly by the midgut suggests that the Type B pump also occurs in the midgut but at a very low density. Alternatively, it is possible that the Type A pump is not totally selective for acid dyes.

The evidence presented above shows that the midgut of larvae of *Manduca* is able to excrete dyes efficiently and is, in fact, the major organ for the excretion of acid dyes. This dye pump in the midgut exhibits all the properties of an active transport mechanism: it excretes dyes against a concentration gradient; it is specific for certain substances; and it is inhibited by DNP, indicating a requirement for ATP as an energy source. In contrast to the situation in previously studied insect species (Palm, 1952) the accumulation of acid dyes in the midgut of *Manduca* larvae is quite dramatic, while the contribution of the Malpighian tubules to the excretion of these dyes appears to be negligible. Although Palm (1952) did not encounter a single case of primary excretion by the midgut, in over 125 species of insects, it is difficult to believe that *Manduca sexta* is the only species in which this phenomenon occurs. The fact that excretion by the midgut has not been noted previously is likely to be due to the general paucity of studies in this area. It is probable that an extensive survey of dye excreting mechanisms in insects would encounter further cases of excretory activity of the midgut and possibly of other organs yet unsuspected.
Excretion by midgut in Manduca

In vitro experiments have shown that the rate of amaranth excretion by pieces of isolated midgut was approximately $9.9 \times 10^{-8} \text{M/h}$ for the entire animal. This figure compares favourably with the mean rate of amaranth excretion in vivo. One and a half micromoles of amaranth ($75 \mu l$ of a $2 \times 10^{-2} \text{M}$ solution) are completely cleared from the haemolymph in about 3 h. This requires a mean rate of excretion of $4.9 \times 10^{-8} \text{M/h}$.

Not all dyes tested appear to be excreted at the same rate. Experiments by Maddrell, Pilcher & Gardiner (1969) suggest that in *Rhodnius* the Malpighian tubules excrete only the anionic form of the acid dye indigo carmine (= indigo sulphonate). Therefore, the rate of dye excretion probably depends on the pK of the dye and the pH of the medium; a higher rate of excretion resulting under conditions which cause greater dissociation of the dye. This could explain the differences observed in the rates of excretion of different dyes (Table 1).

Competition between acid dyes and hippuric acid was evident for the midgut and Malpighian tubules, both in vivo and in vitro. Isolated midguts containing $10^{-2} \text{M}$ amaranth (section 5b of Results) excreted this dye at about the same rate as guts filled with dye at twice that concentration (section 3 of Results). This suggests that, at the concentrations used, the dye transporting mechanism is saturated and dye is being excreted at a maximum rate. In the presence of $0.1 \text{M}$ hippurate the rate of amaranth excretion was decreased to about 60 % of its control level.

The competition between acid dyes and hippuric acid is of particular interest because it clearly suggests the primary role of the dye pump, namely, the elimination of toxic aromatic residues (Smith, 1951; Maddrell, 1971). In addition, the fact that only acid dyes compete with hippuric acid provides further support for the presence of two types of pumps, one to excrete large anions (acid dyes and hippurate) and one for cations (basic dyes).

Finally, larvae of *Manduca* are parsimonious in the use of their dye excreting mechanism. These animals cease to feed shortly prior to the gut purge. In the absence of further ingestion of toxic plant substances a highly efficient excretory mechanism is no longer needed. About 12-24 h after the gut purge the midgut begins to lose its ability to excrete dyes. The Malpighian tubules retain their full activity slightly longer. By 60 h after the purge the midgut no longer excretes dyes and the Malpighian tubules do so at a much decreased rate.

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