

Calcium transport from Malpighian tubules to puparial cuticle of *Musca autumnalis**

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Summary. The transport of calcium from mineralized granules stored in the Malpighian tubules to the puparium of the face fly, *Musca autumnalis* De Geer, was studied. Calcium was transported directly from the tubules to the cuticle via the hemolymph. Little, if any, calcium entered the hindgut or other tissues during or prior to transport. A total of approximately 0.8 mg of calcium per larva was transported, beginning at the wandering stage; peak hemolymph concentrations occurred at anterior retraction. Hemolymph calcium levels subsequently decreased as puparial calcium increased. Puparial mineralization utilized most of the minerals stored during the larval stage, with lesser amounts of minerals being recovered in the adult or excreted. Deposition of mineral salts in the cuticle was accompanied by an increase in cuticular pH from 7.0 to 8.4. The house fly, *Musca domestica* L., which contains much lower concentrations of minerals in the puparial cuticle, exhibited no increase in cuticular pH during pupariation. Biomineralization of the face fly puparial cuticle appears to occur, in part, as a result of ionic equilibria involving calcium and magnesium phosphates and carbonates, which have relatively low solubility products at alkaline pH.

Introduction

Mineralization of insect cuticle, as a method of stabilization, occurs in relatively few insect species,

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most of which are in the order Diptera (Keilin 1921; Eastham 1925; Gilby and McKellar 1976). The face fly, *Musca autumnalis* De Geer, is one species that mineralizes its puparial cuticle with calcium and magnesium salts (Fraenkel and Hsiao 1967; Darlington et al. 1983; Grodowitz and Broce 1983). The face fly stores mineralized granules, which are composed primarily of calcium, magnesium, phosphate and carbonate, together with other minor components in the lumen of the larval Malpighian tubules (Darlington et al. 1983; Grodowitz et al. 1987). The cuticle begins to be mineralized at pupariation. Mineral transport from the tubules, through the hemolymph to the integument for the purpose of cuticle mineralization, has been proposed (Darlington et al. 1983, 1985; Grodowitz and Broce 1983; Roseland et al. 1985; Krueger et al. 1987). Other studies indicate that pH plays a major role in effecting granule dissolution (Krueger et al. 1987). However, little direct evidence is available concerning the movement of dissolved mineral stores through the insect and the role of the hemolymph in its transporation. The objectives of this research were (1) to determine the route and timing of calcium transport from the Malpighian tubules to the puparial cuticle and (2) to determine the distribution of mineral stores between the tubules, puparium, adult fly, and meconium. The role of cuticular pH in mineralization was also examined. The house fly, *M. domestica*, does not mineralize its puparium extensively, and was used for comparative purposes in some experiments.

Materials and methods

Insects. *Musca autumnalis* and *M. domestica* were obtained from colonies maintained in the Department of Entomology at Kansas State University (KSU). Flies were reared at 27±1 °C and 50% r.h. with a 16L:8D photoperiod. Adults were fed water, sucrose and powdered chicken egg. Fly eggs were obtained by introducing 9-cm petri dishes of fresh bovine

feces into cages with 6-day old adults. After 24 h, first larval instars were transferred to larger pans of feces and allowed to develop. Third instars were used for experiments at development stages defined as follows:

D2 and D3 = days 2 and 3 when larvae are actively feeding and beginning to accumulate large quantities of minerals in the Malpighian tubules.

PW1 and PW2 = early and late prewandering stages of day 4 when larvae are still feeding.

W1 and W2 = early and late wandering stages when larvae cease feeding and leave the fecal pat. The maximum weight of the Malpighian tubule contents occurs at this time (Grodowitz and Broce 1983).

AR = anterior retraction when larval cuticle is retracting into a barrel-shaped puparium and mouthparts are still movable.

P1, P2 and P3 = early, mid, and late puparial stage when cuticular mineralization is in progress. P1, P2 and P3 have a duration of about 6, 6, and 12 h, respectively. P3 ends at larval-pupal apolysis.

A = apolysis when the puparial cuticle separates from the epidermis. This occurs on day 5, about 20–30 h after wandering behavior ceases.

Calcium transport. Hemolymph was withdrawn from larvae by puncturing the cuticle lateral to the mouthparts with a 0.15 mm diameter minuten pin attached to a 5- μ l capillary tube. Hemolymph flowed directly from the wound into the capillary tube.

The concentrations of calcium, magnesium, potassium and phosphorus in hemolymph were determined in *M. autumnalis* and *M. domestica* larvae, which were reared in the same fecal sample using the procedure of Grodowitz and Broce (1983). Hemolymph samples from individual larvae were pooled and analyzed for inorganic elements by plasma emission spectroscopy at the KSU Emission Spectroscopy Laboratory.

For mineral budget experiments, eight separate groups of 100 larvae were reared in pans containing 1.2 kg feces. Pupae from these groups were placed singly in gelatin capsules. After emergence, the puparial exuvia, adult, meconium and first feces from individuals were pooled, ashed, solubilized in 1 M HCl and analyzed for calcium, phosphorus, magnesium and potassium by plasma emission spectroscopy.

In radiotracer experiments, first instar larvae were introduced into bovine feces (1 larva per 3 g feces) prepared by mixing 550 g fresh feces with 50 ml water containing 150 μ Ci $^{45}\text{CaCl}_2$ (0.25 $\mu\text{Ci}\cdot\text{g}^{-1}$, New England Nuclear). After 3 days, groups of 50 larvae were transferred to nonradiolabeled feces at 10, 9, 7, 6, 4, 3, and 1 h before wandering. Times were estimated according to cuticular coloration, gut content and time of first instar transfer. Another group of larvae was allowed to develop to wandering in the radiolabeled feces. Radioactivity was determined after dissolving hemolymph samples in a liquid scintillation cocktail (Fisher ScintiVerse I).

^{45}Ca was also used to determine the amount of calcium present throughout development in the Malpighian tubules, cuticle, and remaining tissues rinsed of hemolymph. First larval instars were transferred to feces labeled with 0.08 $\mu\text{Ci } ^{45}\text{Ca}\cdot\text{g}^{-1}$ and allowed to remain until wandering. At different stages of pupariation, tissues were dissected, pooled and homogenized in 0.1 N HCl. The radioactivity of an aliquot of the supernatant was counted and the remainder analyzed for total calcium by atomic absorption spectroscopy at the KSU Animal Nutrition Laboratory.

Cuticular pH. The pH of the cuticle was measured with a flat-surface microelectrode with a 1 mm sensing diameter (Microelectrodes Inc., Londonderry, NH). Posterior abdominal cuticle

was dissected from larvae, rinsed briefly in deionized water, and pinned to a wax dish. Epidermis was scraped from the internal cuticular surface as quickly as possible. The reference and microelectrode were then lowered onto either the internal or external surface of the cuticle.

Statistical analysis. Statistical tests of significant differences between stages and statistical differences from zero were performed using the SAS least-squares means procedure (LSMEANS) with $P \leq 0.05$ (Statistical Analysis Systems Institute, Inc., Cary, NC).

Results

Mineral distribution during pupariation and adult development

The calcium content of the Malpighian tubules of *Musca autumnalis* was inversely related to that of the puparial cuticle during the larval-pupal transformation, suggesting that mineral salts are transported from the former to the latter (Fig. 1). Tubule calcium decreased from more than 0.8 mg at early wandering (W) to less than 0.1 mg at approximately 8 h prior to apolysis (P3). Cuticular calcium increased after anterior retraction and approximately 0.6 mg was deposited by the end of pupariation. Other tissues, including fat body, gut and

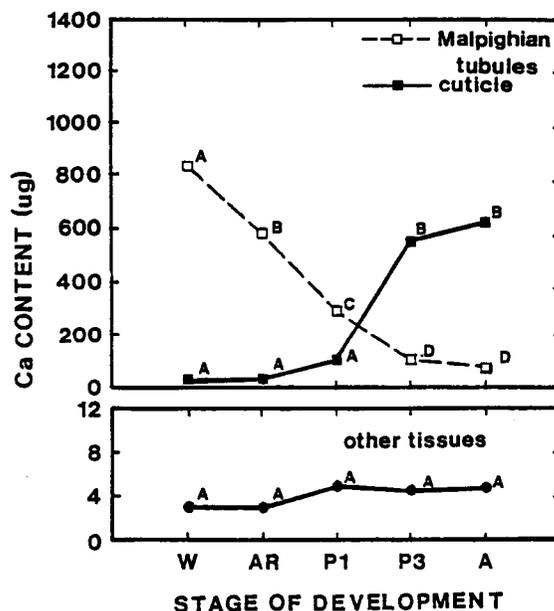


Fig. 1. Total Ca (μg) in the Malpighian tubules, cuticle, and other tissues of individual *Musca autumnalis* during the larval-pupal transformation. 'Other tissues' include the gut, fat body, and tracheal system that were rinsed of hemolymph. $n = 3$. Letters denote significant differences in calcium concentration within tissues, $P \leq 0.05$. Means with the same letter are not significantly different. See Materials and methods for staging nomenclature.

Table 1. Total minerals (μg) in various tissues and excretory products after adult eclosion of *Musca autumnalis*

Tissue	Mineral content ^a			
	Calcium	Magnesium	Phosphorus	Potassium
Puparium	630 \pm 24 (77)	145 \pm 6 (83)	432 \pm 23 (82)	76 \pm 8 (30)
Meconium	37 \pm 16 (5)	9 \pm 5 (5)	10 \pm 2 (2)	9 \pm 1 (4)
Adult fly	96 \pm 8 (12)	19 \pm 2 (10)	80 \pm 7 (15)	91 \pm 6 (35)
First feces	60 \pm 10 (7)	3 \pm 1 (2)	8 \pm 2 (2)	81 \pm 18 (32)
Total	823 (101)	176 (100)	531 (101)	258 (101)

^a Units = μg per fly \pm SE, $n = 3$ (number of analysis of pooled tissues). Percent of total given in parenthesis

trachea, contained little calcium and exhibited no significant change during pupariation (Fig. 1). These data show that from W to P1 (ca. 8 h) calcium was removed from the tubules at a relatively constant rate of about $1 \mu\text{g} \cdot \text{min}^{-1}$. This estimated rate declined to $0.04 \mu\text{g} \cdot \text{min}^{-1}$ over the 12 h period between P1 and A. Calcium deposition in the puparium was relatively slow initially. Subsequently, a rapid rise occurred between P1 and P3, until maximum content was obtained. Thus, calcium is translocated from the Malpighian tubules to the cuticle between wandering and apolysis, with the bulk of transport occurring after anterior retraction.

A majority of the calcium, magnesium, and phosphorus was deposited in the puparium, with only minor quantities found in other tissues and excretory products after adult eclosion (Table 1). Approximately 80% of these elements remained in the puparium after adult eclosion and 5% or less was excreted in the meconium. The remaining 15% was found in adults and their excretory material. Potassium was distributed almost equally between the puparium, adult and waste products.

Mineral composition of hemolymph

To establish whether the cuticular mineral salts are transported in the hemolymph of *M. autumnalis*, serum mineral content was determined during pupariation. For comparison, the hemolymph from *M. domestica*, a species that sclerotizes rather than mineralizes the puparium, was analyzed. Phosphorus, magnesium and calcium concentrations were 1.7–2.0 times higher in *M. autumnalis* third instar hemolymph than in *M. domestica* hemolymph (Ta-

Table 2. Mineral composition of hemolymph from third instar *Musca autumnalis* and *M. domestica**

Mineral	Mineral concentration (g/l)	
	<i>M. autumnalis</i>	<i>M. domestica</i>
Phosphorus	1.07 \pm 0.08 ^A	0.53 \pm 0.15 ^B
Magnesium	0.53 \pm 0.05 ^A	0.32 \pm 0.05 ^B
Calcium	0.42 \pm 0.06 ^A	0.25 \pm 0.07 ^B
Potassium	1.18 \pm 0.11 ^A	1.02 \pm 0.10 ^A

Groups of larvae were reared in subsamples of bovine feces from the same source. Mean \pm SE, $n = 6-8$ (number of analysis of pooled tissues), letters denote significant differences between species, $P \leq 0.05$

ble 2). Potassium levels were similar in both species. Prior to wandering, the hemolymph calcium concentration in *M. autumnalis* was relatively constant, varying from 0.35 to $0.40 \text{ g} \cdot \text{l}^{-1}$ (Fig. 2). However, a twofold increase occurred at late wandering to anterior retraction and was subsequently followed by a rapid decline to levels similar to those observed prior to wandering. These fluctuations in hemolymph calcium correlated well with the decrease in calcium in the Malpighian tubules, which occurred before appreciable calcium deposition in puparial cuticle.

Calcium transport in hemolymph

Measurement of hemolymph calcium concentration does not prove that mineral transport by hemolymph occurs between the Malpighian tubules and the puparial cuticle. It also does not give an accurate indication of the timing of calcium release from the anterior Malpighian tubules. Thus, a la-

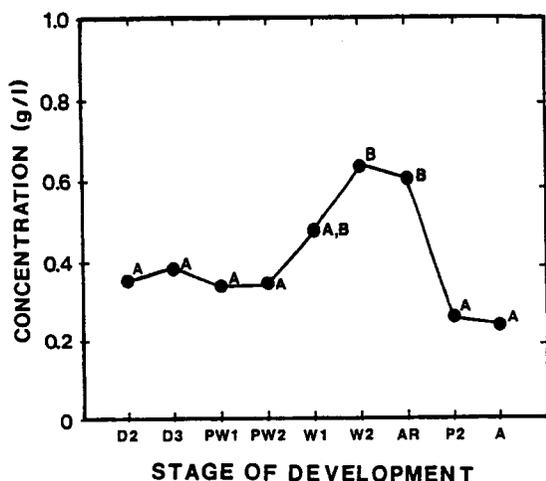


Fig. 2. The concentration of Ca ($\text{g}\cdot\text{l}^{-1}$) in *Musca autumnalis* hemolymph during larval-pupal development. $n=3-5$. Letters denote stage-specific differences in calcium concentration, $P\leq 0.05$. Means with the same letter are not significantly different. See Materials and methods for staging nomenclature

being study using ^{45}Ca was conducted to investigate transport. ^{45}Ca levels were measured in larvae that were reared continuously in medium containing this isotope (Fig. 3A) and in larvae that were transferred to unlabeled medium for variable time periods prior to wandering (Fig. 3A, B). The experiment was conducted to determine (1) the time required to clear hemolymph of ^{45}Ca , (2) the time of ^{45}Ca release from the Malpighian tubules, and (3) the difference in timing of ^{45}Ca release from Malpighian tubules in larvae pulsed for various time intervals. The latter investigation would reveal any relationship between calcium accumulated and calcium released. For example, if larvae pulsed for 10 h released ^{45}Ca later than larvae pulsed for 4 h, then it would appear that calcium sequestered last from the hemolymph (which would be unlabeled) was released first during cuticular mineralization. No differences in initial release time would indicate that calcium sequestered last was also released last. In addition, by pulsing for variable time intervals, the actual time of calcium release can be more accurately predicted.

The radiolabeling results indicated that larvae reared in ^{45}Ca -labeled feces and removed to unlabeled feces at various times prior to wandering behavior had hemolymph calcium increases of similar magnitude between W1 and early AR (Fig. 3A, B). The initial increase of ^{45}Ca in hemolymph occurred at about the same time for all larvae, irrespective of the time spent in unlabeled feces. For example, data obtained using larvae held in unlabeled feces for time intervals as long as 10 h before wandering were essentially the same as those held

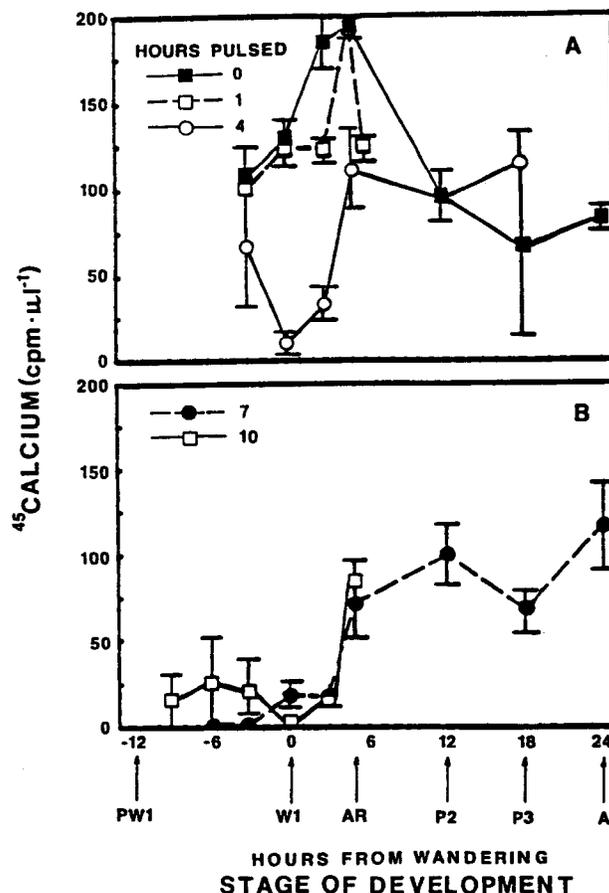


Fig. 3. ^{45}Ca activity ($\text{cpm}\cdot\mu\text{l}^{-1}$) of *Musca autumnalis* hemolymph from larvae grown in radioactive feces (hours pulsed = 0) and from larvae transferred to nonradioactive feces for variable lengths of time before wandering (10, 7, 4 and 1 h). $n=3-5$. See Materials and methods for staging nomenclature. Values are mean \pm SE

for 4 and 7 h. When larvae were transferred to unlabeled feces 1 h prior to wandering, ^{45}Ca levels in the hemolymph were similar to those in non-pulsed larvae, but quite different from levels in pulsed larvae. By the later stages of development (P2, A), there was no significant difference detected in hemolymph transport of ^{45}Ca in pulsed and non-pulsed larvae. Also, in related experiments, no ^{45}Ca was detected in the hindgut of either pulsed or non-pulsed larvae, even when specific activity of the label was increased fourfold from $0.25\ \mu\text{Ci}\cdot\text{g}^{-1}$ to $1.0\ \mu\text{Ci}\cdot\text{g}^{-1}$.

pH of cuticle during pupariation

Mineral deposition in the Malpighian tubules of the face fly is associated with an alkaline pH (Krueger et al. 1987). In order to determine if mineralization of the puparial cuticle also occurs under basic conditions, microelectrodes were used to re-

Table 3. The pH of the outer and the inner surfaces of larval cuticle during pupariation of *Musca autumnalis* and *M. domestica*

Stage ^a	Surface pH*			
	<i>M. autumnalis</i>		<i>M. domestica</i>	
	Outer pH	Inner pH	Outer pH	Inner pH
W	7.04 ± 0.13 ^A	7.20 ± 0.19 ^A	6.85 ± 0.10 ^A	6.88 ± 0.09 ^A
AR	7.47 ± 0.12 ^B	7.60 ± 0.14 ^B	6.75 ± 0.15 ^A	7.00 ± 0.13 ^A
P1	8.42 ± 0.12 ^C	7.90 ± 0.20 ^B	6.72 ± 0.18 ^A	6.55 ± 0.10 ^B
P3	8.18 ± 0.22 ^C	7.80 ± 0.06 ^B	6.82 ± 0.20 ^A	7.00 ± 0.15 ^A
A	8.38 ± 0.04 ^C	8.43 ± 0.14 ^C	7.05 ± 0.15 ^B	7.20 ± 0.10 ^C

* Mean pH ± SE, $n=3-4$ (number of analysis of pooled tissues), letters denote significant differences ($P \leq 0.05$) within a stage.

^a See text for stage definitions

cord the surface pH of larval cuticle during pupariation (Table 3). In *M. autumnalis*, the pH of both the inner and outer surfaces of the puparial cuticle was about 7.1 at early wandering. At anterior retraction both cuticular surfaces had a pH near 7.5, after which the outer surface increased to pH 8.4 during early pupariation (P1). It is during P1 that mineralization becomes visibly evident, with the cuticle beginning to turn opaque white. The inner surface pH increased more slowly and did not attain pH 8.4 until apolysis. In contrast, the brown sclerotized puparial cuticle of *M. domestica* was more acidic (pH 6.6–7.2) and the pH of its two surfaces remained near pH 7 during sclerotization (Table 3). Another highly sclerotized cuticle, that of the pupa of the tobacco hornworm (*Manduca sexta*), had a pH that also varied from only pH 6.6–7.1 during the process of sclerotization (Krueger, unpublished data). Thus, an increase in pH occurs during mineralization but not during sclerotization of the cuticle.

Discussion

Results from the analysis of calcium concentrations in tissues and from experiments with radiolabeled calcium demonstrated that calcium is transported from the Malpighian tubules to the puparial cuticle directly through the hemolymph. The lack of radioactive calcium in the hindgut also supported this hypothesis. While substantial amounts of calcium (0.5–1.0 mg) were translocated and a 20-fold increase in puparial content occurred within a period of 24–30 h, only a twofold increase in calcium concentration occurred in the hemolymph during that period. In addition, phosphorus, magnesium and calcium were only about twofold higher in face fly third larval instars than in

house fly larval instars. Presumably, the relatively small fluctuations in mineral concentration in face fly hemolymph reflect an almost steady-state regulation, such that transport into and out of the hemolymph increases without large changes in ionic levels. The hemolymph concentrations of minerals in both species are similar to those of other dipterous larvae (Florkin and Jeuniaux 1974).

Calcium transport in hemolymph was first observed in pulsed larvae at W2 to AR. During and following those stages, a decrease in Malpighian tubule calcium was followed by an increase in puparial calcium. The source of calcium in the puparium, therefore, must be that released from storage granules in the lumen of the tubules, as suggested by Grodowitz and Broce (1983). It was noted that radiolabeled calcium was detected in the hemolymph during the same time period in all pulsed larvae, regardless of the number of hours they had been feeding in non-radiolabeled feces. This suggests that the mineralized granules formed last in the most distal region of the tubule lumen were also the last to be dissolved. The older granules probably move into the more acidic region of the Malpighian tubules first. If this were not so, larvae that were in unlabeled feces for 10 h would have exhibited a delayed increase in hemolymph ⁴⁵Ca compared to larvae that were pulsed for only 4 h. The lack of a pulsing effect supports the hypothesis that formation of granules occurs in the distal region of the Malpighian tubules, with dissolution of granules and release of minerals into the hemolymph occurring in the proximal region (Grodowitz and Broce 1983; Krueger et al. 1987).

Musca autumnalis apparently transports minerals through the hemolymph to the cuticle in a well regulated manner. The greatest change in calcium titer in the hemolymph was ca. 2-fold. Regulation

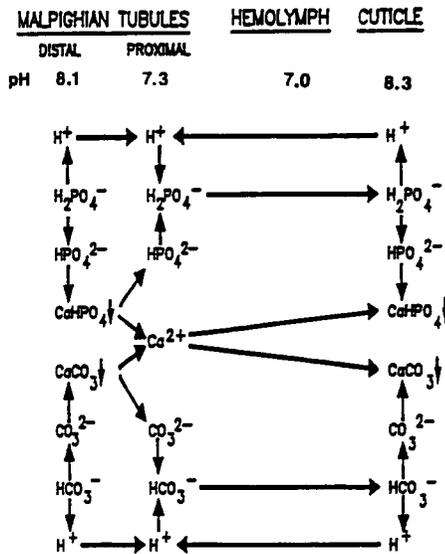


Fig. 4. A hypothetical scheme showing the effect of pH on dissolution, transport, and precipitation of calcium salts in Malpighian tubules, hemolymph, and cuticle of *Musca autumnalis*. The major salt is probably CaHPO_4 .

of calcium transport and subsequent mineralization may be accomplished by factors which affect (1) the rate of dissolution and transport of minerals from the Malpighian tubules, (2) the rate of transport of minerals through the hemolymph, or (3) the uptake of minerals by epidermal cells and deposition in cuticle. One factor that may regulate deposition of minerals in *M. autumnalis* cuticle is pH. Cuticular pH is important, since it may determine the physical and ionization states of minerals and organic functional groups, as well as the reactivity of enzymes and metabolic intermediates involved in cuticle morphogenesis. The alkalinity of the puparial exoskeleton of *M. autumnalis* at the onset of mineral deposition is similar to that in the distal region of the Malpighian tubules, where mineralized granules are probably formed (Krueger et al. 1987). This suggests that in both the Malpighian tubules and epidermis, *M. autumnalis* may regulate mineralization via a proton translocation mechanism that influences ionic equilibria of mineral salts, as dictated by ionization constants and solubility products. Even though we measured cuticular pH using a surface microelectrode, the increase in pH during puparial mineralization was comparable to that observed in crab carapace measured with a radiolabeled pH marker procedure (Wood and Cameron 1985). The internal pH may be somewhat altered by the scraping of the epidermis, but it did not appear to be altered by the degree of scraping, suggesting that the ob-

tained values may be quite valid. Similar phenomena of pH regulation have been observed in studies of carapace mineralization by the blue crab, *Callinectes sapidus* (Wood and Cameron 1985; Cameron and Wood 1985) and the shore crab, *Carcinus maenas* (Digby 1985). During carapace mineralization, cuticular pH appeared to be raised by extruding hydronium ions or accumulating hydroxide ions, respectively, in order to facilitate the precipitation of calcium carbonate.

The processes of calcium uptake and deposition appear to occur readily under alkaline conditions in both insect and crab cuticles. However, the major salt in *M. autumnalis* cuticle is calcium phosphate (Darlington et al. 1983), while in the crustacean cuticle it is calcium carbonate. A hypothetical scheme for the interaction of pH, ionic equilibria, mineral states and transport processes in *M. autumnalis* tissues during pupariation is shown in Fig. 4. In the hemolymph, which has a pH of 7.0 (Krueger et al. 1987), calcium, magnesium, dihydrogen phosphate and bicarbonate would be the main ions present. Transport of those ions into the integument, together with an increase in cuticular pH, would then cause dissociation of dihydrogen phosphate and bicarbonate ions into monohydrogen phosphate, carbonate and hydrogen ions. The anions would combine with cations, precipitating CaHPO_4 , MgHPO_4 , CaCO_3 and MgCO_3 in the exoskeleton, since all of those salts have low solubility product constants ($K_{sp} < 10^{-5}$). The indication that the associated release of hydrogen ions during mineral deposition does not lower the pH of the cuticle suggests that the epidermis has either a substantial buffering mechanism, or an active transport system that shuttles protons from the exoskeleton to the hemolymph. Such a transport system would provide a mechanism for making the cuticular fluid alkaline, thus enhancing precipitation of mineral salts. Thus, the inorganic process of mineralization in the puparial cuticle of *M. autumnalis* and in crab exoskeleton occurs at an alkaline pH (8.3), whereas the organic process of cuticle strengthening or sclerotization in *M. domestica* and *Manduca sexta* occurs at neutral pH.

In conclusion, minerals from the Malpighian tubules are transported to the cuticle via the hemolymph. The dissolution and translocation of mineral stores appear to be initiated in late wandering by 20-HE, which also initiates pupariation (Fraenkel and Hsiao 1967). Deposition of minerals in the puparium is completed between 24 and 30 h later. The hindgut and other tissues are not involved in this translocation process. Most of the calcium and magnesium salts stored in larval Mal-

pighian tubules are used later for puparial mineralization, with minor carryover to the adult fly. The calcium concentration in the hemolymph doubles when minerals are released from the tubules, just before deposition begins in the cuticle. It decreases to relatively low levels over the next few hours, as the puparial cuticle is mineralized. The deposition of minerals in the cuticle is associated with an increase in pH from 7.0 to 8.4, which may be the driving mechanism by which mineral deposition is induced, since in an alkaline environment, phosphate and carbonate salts of calcium and magnesium are very insoluble.

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