MINERAL MOBILIZATION FROM THE MALPIGHIAN TUBULES FOR HARDENING OF PUPARIAL CUTICLE IN THE FACE FLY, *Musca autumnalis* De Geer

by

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DEDICATION

This thesis is dedicated to the memory of my mother, Beverly A. Reijo, whose life taught me the value of education and hard work and whose support, encouragement, and unconditional love are now so greatly missed.

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LITERATURE REVIEW

The face fly, Musca autumnalis DeGeer, is one of a few insects to mineralize its puparium. Larvae of the face fly accumulate calcium and other minerals in the form of granules or spheres in the anterior Malpighian tubules. These granules consist primarily of calcium, phosphate, magnesium, and carbonate with minor amounts of potassium also present (Darlington et al., 1983). The granules are stored in the Malpighian tubules through the wandering stage of the larvae (the stage when the larvae leave the fecal pat and cease feeding). The disappearance of the granules from the Malpighian tubules is initiated shortly after wandering and has been correlated with the onset of mineral deposition in the face fly puparium (Grodowitz and Broce, 1983). The minerals, therefore, must be dissolved from the granules prior to their transport to the puparium. This review focuses primarily on literature related to possible mechanisms of granule dissolution and the route of minerals from the Malpighian tubules to the puparium.

Although the face fly is one of the few insects to mineralize its puparium, the occurrence of granules in the excretory organs and tissues, both extracellularly and intracellularly, is widespread in invertebrates. Granules occur in species of annelid worms (Gibbs and Bryan, 1984), in fresh- and salt-water clams (Marsh and Sass, 1983), mussels (Petit et al., 1980), snails (Abolins-Krogis, 1970; Howard et

al., 1981; Burton, 1972; Fournie and Chetail, 1982; Watabe et al., 1976), crabs (Guary and Negul, 1981; Hopkin and Nott, 1979), other crustaceans (Travis, 1950), and many insects. Among the insects, granules are found in Collembola (Humbert, 1978), Orthoptera (Simkiss, 1976), Hemiptera (Clark, 1958; Wigglesworth and Salpeter, 1962), Homoptera (Marshall and Cheung, 1973), Coleoptera (Knutson et al., 1967), Lepidoptera (Tiegler and Arnott, 1972; Turbeck, 1974; Waku and Sumimoto, 1974) and many Dipterans, including the face fly (Fraenkel and Hsiao, 1967; Darlington et al., 1983; Grodowitz and Broce, 1983), house fly (Sohal et al., 1976; Grodowitz and Broce, 1983), and others (Eastham, 1925; Keilen, 1921; Waterhouse, 1950; Gilby and McKeller, 1976). General reviews of the metal-containing granules in invertebrates have been conducted by B. E. Brown (1982) and K. Simkiss (1976).

Mineralized granules accumulated in invertebrate tissues are generally attributed any one or more of the following functions:

1. The granules may be involved in detoxification mechanisms. Precipitation of toxic materials in the granules removes the toxics from the ionic pool. Sohal et al. (1977) and Simkiss (1976) have proposed this function.

2. The granules may be merely a method of removing excess ions from the metabollic pool for later excretion as suggested by Waterhouse (1950) in the case of Lucilia cuprina.

3. The granules may serve as a reserve or storage mechanism for calcium and/or other minerals to be utilized later in development.

The face fly, as well as several other dipterans, are thought to utilize the granules for cuticular hardening (Keilen, 1921; Gilby and McKellar, 1976; Grodowitz and Broce, 1983; Roseland et al., 1985). Other uses include deposition in developing ova (Taylor, 1984; Shaw and Stobbart, 1963) or hardening of various plates or septa as in the snail-killing flies (Knutson et al., 1967) or Cerambyx larvae (Clark, 1958). In the molluscs, crustaceans, and gastropods, granules are believed to be utilized for reproduction requirements (Fournie and Chetail, 1982) or in shell regeneration and calcium mobilization (Becker et al., 1974; Hopkin and Nott, 1979; Abolins-Krogis, 1970; Watabe et al., 1976; and others). Though utilization of these mineral reserves is well documented in the literature little is known about the dissolution mechanisms or transport of the ions.

Grodowitz and Broce (1983) suggested that the dissolution of the face fly granules may be enzymatic or related to a pH change in the proximal portion of the Malpighian tubules. They supported this theory with photographs of granules isolated from the proximal Malpighian tubules showing signs of natural dissolution. Later, Darlington et al. (1984) suggested that carbonic anhydrase secreted into the hemolymph by the posterior midgut, near the point of attachment of the Malpighian tubules, may effect a pH change in the proximal portion of the Malpighian tubules and bring about granule dissolution. However, they did not find measurable carbonic anhydrase activity in close contact with the granules in the

Malpighian tubules. Thus, the role of carbonic anhydrase in actual mobilization of the mineral reserves in face fly larvae, as Istin and Girard (1970) found in several lamellibranchs, remains equivical. Turbeck (1974) found that the granules or spherites of the midgut cells in a Lepidopteran larvae disappeared at a time when they would be biologically useful in cell growth and suggested a decrease in the intracellular pH caused the dissolution of the granules and the release of the compounds contained in them.

Additional support for the hypothesis that a decrease in pH in a distinct region of the Malpighian tubules may occur and effect granule dissolution in the face fly is found in literature relating to insect Malpighian tubule function. Wigglesworth (1931) described the Malpighian tubules of Rhodnius prolixus and related structural variations in histology to physiological events. In Rhodnius each tubule can be divided into two regions, proximal and distal. Wigglesworth (1931) concluded that the entire distal portion of the tubules does not contain crystals of uric acid but apparently functions in excretion. The proximal portion functions in reabsorption of water; and crystals are found here due to this concentrating effect. The pH of the proximal portion, he noted, did decrease where the Malpighian tubules empty into the gut. Stobbart and Shaw (1974) and Cochran (1975) also noted this occurrence in the proximal tubule portion in some insects. Waterhouse (1950) recorded a slight decrease in pH in the proximal tubules in Lucilia cuprina.

Wigglesworth and Salpeter (1962) cited further

information on the functions of the proximal and distal segments of the Malpighian tubules. They considered the distribution of mitochondria as evidence of the proximal portion's reabsorption function and the uptake function of the distal portion.

Srivastava (1962) stated that on the basis of morphology and location the Malpighian tubules of Corcyra cephalonica can be divided into three segments: 1) Distal, 2) middle, and 3) proximal. He concluded that no substance from the hemolymph enters the proximal portion, the middle portion functions in the uptake (or absorption of water and ions from hemolymph) and the distal portion functions only in water uptake bringing about the flushing of the tubule contents into the gut. Srivastava (1962) also suggested that each section of tubule had only a one-way permeability.

Sohal (1974) reported that the Malpighian tubules of the house fly, M. domestica, a close relative of the face fly, are composed of four cell types. The main segments of the Malpighian tubules are composed of cell types I, II, and III intermingled. The proximal portion of the Malpighian tubules is composed of cell type IV only. This suggests a different function for this section of Malpighian tubule. More recently, however, Maddrell (1980) has urged caution in assuming that structure/location and function are correlated. Cells or regions that structurally are similar may function differently and vice versa. Nevertheless support for the theory that different regions of the Malpighian tubules function

differently persists in the literature. Thus, insects, including face fly larvae, may use solubility properties as a function of pH to either store or mobilize mineral salts in a tissue such as the Malpighian tubules.

Additionally, the Malpighian tubules' function may be affected by developmental changes in the insect. Ryerse (1978, 1980) presented evidence that secretion of the Malpighian tubules of Calpodes ethlius is terminated at the onset of pupation due to the presence of 20-hydroxyecdysone. Waterhouse (1950) reported that the larval calcium salts accumulated in the Malpighian tubules of Lucilia cuprina are not excreted until adult emergence. Also Eastham (1925) noted that in Drosophila funebris, the tubules change in cellular appearance at pupation, regain a form similar to the larval form six days later, and pass the calcium carbonate stored within the Malpighian tubules to the midgut. Fraenkel and Hsiao (1967) determined that the calcification of the face fly is dependent on ecdysone. Thus, a working hypothesis based on published evidence would be as follows: The face fly may accumulate granules through the larval stages, secretion to the midgut-hindgut region by the tubules may be discontinued by the action of ecdysone at pupariation, and the granules may dissolve in the proximal portion of the Malpighian tubules due perhaps to a pH change that may be enzymatically controlled.

Waterhouse (1950) also observed in in vitro studies that the crystals of calcium phosphate collected from Lucilia cuprina larvae were soluble in dilute acids but not in alkali, any common organic solvent, or water. Waku and Sumimoto

(1971) noted the same acid solubility profile. Termine and Posner (1970) reported on the factors affecting the initial precipitation of amorphous calcium phosphate. They found that precipitates of amorphous calcium phosphate which take only 10 - 15 min. to form at 25°C and ph 7.7 require 2.3 - 7.5h to form at a pH of 7.4 at the same temperature. These workers also altered other environmental conditions such as ionic strength, viscosity, and ion concentrations of sodium, potassium, magnesium, calcium, phosphate, carbonate, pyrophosphate, and selected macromolecules. All of the above, except sodium and potassium, altered the precipitation time to some degree. From this information a change in pH seems to be an attractive hypothesis for granule dissolution. However, an active transport system alone is a possibility. Berridge and Oschman (1969) suggested that in Calliphora Malpighian tubules, two types of cells are present along the length of the tubules, namely stellate and primary cells. The primary cells may be involved in osmotic water flow and the secretion of ions, whereas the stellate cells are involved in reabsorption of sodium. Dalton and Windmill (1980) found that in M domestica calcium and magnesium are essential for secretion and the rate of secretion is inversely proportional to the osmotic pressure of the isolating medium in in vitro studies. Thus, a number of factors may be interlinked in considering the dissolution of calcium phosphate granules in the face fly.

Once dissolution has occurred, transport of the minerals

from the Malpighian tubules to the puparium must follow or at least be occurring simultaneously. Three possible routes of calcium transport are evident: 1) Transport of the calcium from the tubule into the hindgut and eventual emptying into the ecdysial space; 2) transport of the calcium from the tubule into the hindgut and reabsorption of the ions by the rectum; or 3) transport of the calcium through the tubule membrane directly into the hemolymph.

Eastham (1925) observed peristalsis of the Malpighian tubules in D. funebris with the subsequent passage of granules into the gut. The calcium granules were then passed out of the body through the anus. Tiegler and Arnott (1972) also observed evidence for this type of transport system in the larvae of Bombyx mori. In the silkworm, crystals of calcium oxalate are passed from the Malpighian tubules through the gut and the crystals flow from the anus to the surface of the new cuticle. It is notable in both these cases, that the minerals in the granules were not utilized in cuticular hardening.

Waterhouse (1950) suggested that the second type of transport, namely, movement of the calcium from the tubule into the hindgut followed by reabsorption by the rectum, takes place in the Calliphora larvae. He proposed that calcium granules accumulate in the Malpighian tubules, the permeability of the tubule membrane changes, and water entering the tubule flushes the granules into the hindgut. Reabsorption of useful molecules by the rectum would follow. Dissolution was not documented.

Keilen (1921) observed that during the first five days of

metamorphosis in Acidia and Agromyza larvae, calcium carbonate granules disappear from within the pupae and he presumed that bathing of the granules by the perivisceral fluid effected dissolution. Keilen did not detail the site of dissolution. The dissolved minerals were then resorbed in the puparium.

Weismann (1938), as quoted by Clark (1958), noted that calcium is transported from the Malpighian tubules of Rhagoletis cerasi to the cuticle before the cuticle separates from the epidermal cells. The same evidence was noted by Grodowitz and Broce (1983) in face fly larvae.

From the above evidence, one would expect the calcium may travel through the hemolymph at some point in time. The transported calcium may be in two forms, bound to a protein or other macromolecule and/or as free ions. Carrington and Tenney (1959) confirmed the existance of a calcium-binding protein in Telea polyphemus. Weidler and Sieck (1977) discovered calcium binding in the hemolymph of Periplaneta americana.

Jungreis et al. (1973) noted that in larval Cecropia the hemolymph concentrations of calcium changed little with ontogeny or diet and averaged about 10 meq/l. Shaw and Stobbart (1963) also suggested that a constancy of hemolymph calcium concentrations occurs in many insects. Indeed, the stability of hemolymph calcium concentrations is necessary for proper cellular function and muscular control (Weevers, 1966). The regulation of the hemolymph concentrations of calcium ions

therefore may be affected by dietary calcium, the permeability of the Malpighian tubules or the rate of mineral transport from the tubules, by the presence of some carrier molecule, or by the rate of uptake by the puparium or any combination of these factors.

The objective of this research was to study the process by which granule dissolution occurs as well as the manner and route of mineral transport to the puparial cuticle; these are among the pivotal events in the physiology of face fly mineralization. The examination of these processes in this insect may lead to advances in understanding the role of minerals in other systems.

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PAPER

MINERAL MOBILIZATION FROM THE MALPIGHIAN TUBULES FOR HARDENING OF PUPARIAL CUTICLE OF THE FACE FLY, *Musca autumnalis* De Geer

ABSTRACT

The process of dissolution of mineralized granules stored in the Malpighian tubules of the face fly, Musca autumnalis, and the subsequent transport of inorganic constituents for use in mineralization of the puparium, was examined. Changes in granule morphology induced during in vitro experiments were correlated with in vivo granule changes during developmental events. Measurements of pH of the larval Malpighian tubules, hindgut, hemolymph, and cuticle were taken to determine the role of pH in the physiology of mineralization. The route of calcium from the granules in the Malpighian tubules to the cuticle was also traced.

The pH of the proximal portion of the Malpighian tubules was significantly lower (7.35) than that of the distal portion of the tubules (8.08). In addition, in vitro experiments indicated that a pH decrease of this magnitude resulted in both increased mineral release from the granules and increased morphological damage to the granules. Minerals released from the granules were apparently transported directly from the Malpighian tubules to the cuticle via the hemolymph. The hindgut and rectum were not involved in transport during the larval-pupal transformation stages. A total of 0.6 - 1.0 mg of calcium was transported, in a steady state process, to the cuticle with no significant changes in the calcium concentration or osmolality of the hemolymph. Most of the minerals stored in the larval stages were utilized in the

puparia with minor amounts being utilized in the adult fly, and some excreted as waste. The deposition of minerals in the puparial cuticle was accompanied by an increase of cuticle pH from 7.04 at wandering stage to 8.4 by the early pupal stage.

INTRODUCTION

The face fly, Musca autumnalis DeGeer, is one of a few insect species to mineralize its puparium. Larvae of the face fly accumulate Ca, P, Mg, and CO_3 (Darlington et al., 1983) in the form of granules or spheres in the anterior Malpighian tubules (Grodowitz and Broce, 1983). These granules are stored in the Malpighian tubules through the wandering stage of the larvae (the developmental stage when the larvae leave the fecal pat and cease feeding). The disappearance of the granules from the Malpighian tubules has been correlated with the onset of mineral deposition in the face fly puparium (Grodowitz and Broce, 1983). The minerals, therefore, must be dissolved from the granules prior to their transport to the puparium.

Though the face fly is one of the few insects to mineralize its puparium, the occurrence of granules in the excretory organs and tissues, both extracellularly and intracellularly, is widespread in invertebrates. Mineralized granules are found in such diverse organisms as clams (Marsh and Sass, 1983), snails (Abolins-Krogis, 1970; Howard et al., 1981; Burton, 1972; Fournie and Chetail, 1982; Watabe et al., 1976 and others), several crustaceans (Guary and Negul, 1981; Hopkin and Nott, 1979; Travis, 1950), and in many insects throughout the orders. General reviews of the metal containing granules in invertebrates have been conducted by B.E. Brown (1982) and K. Simkiss (1976).

Frequently utilization of these mineral reserves has been

hypothesized and/or documented. Common uses include: 1) Cuticular hardening (Keilen, 1921; Gilby and McKellar, 1976; Grodowitz and Broce, 1983; Darlington et al., 1983; Roseland et al., 1985; Becker et al., 1974; Hopkin and Nott, 1979; Abolins-Krogis, 1970; Watabe et al., 1976; as well as others), 2) utilization for reproductive needs (Taylor, 1984; Shaw and Stobbart, 1963; Fournie and Chetail, 1982), and, 3) hardening of various plates or septa (Knutson et al., 1967; review by Clark, 1958). Though utilization of these reserves is well-documented, little is known of the dissolution mechanisms leading to subsequent mineral transport.

Grodowitz and Broce (1983) suggested that the dissolution of the face fly granules may be enzymatic or related to a pH change occurring from the distal to the proximal portion of the Malpighian tubules. They supported this hypothesis with evidence of dissolving granules found only in the proximal portion of the Malpighian tubules. Similar dissolving granules were observed by Watabe et al. (1976) in Pomacea paludosa. Later, Darlington et al. (1984) suggested that carbonic anhydrase found to increase at wandering in the posterior midgut (near the point of attachment of the Malpighian tubules) may be producing hydronium ions that are released into the hemolymph. These hydronium ions could then be passed into the proximal portion of the Malpighian tubules and affect dissolution. Direct evidence of carbonic anhydrase involvement in the dissolution mechanism, as Istin and Girard (1970) found in the dissolution mechanism in several lamellibranchs, has not been shown, however. Darlington et

al. (1984) did not find considerable carbonic anhydrase activity in the Malpighian tubules. Other research indicates that the proximal portion of the Malpighian tubules in some insects may function differently from the distal portion (Sohal et al., 1974; review by Stobbart and Shaw, 1974) and that a pH change from the distal to the proximal portion may occur (review Stobbart and Shaw, 1974).

The objectives of this research, therefore, were to: 1) Elucidate more clearly some of the factors affecting granule dissolution, primarily the role of pH, through in vitro experiments, 2) determine the pH of the Malpighian tubules, hemolymph, hindgut, and cuticle, and, 3) trace the route of minerals from the Malpighian tubules to the puparium.

MATERIALS AND METHODS

Face flies were obtained from colonies maintained in the Department of Entomology at Kansas State University. Flies were reared in a room held at 27°C and 50% RH. Approximately 500 pupae were placed in an 18.5X30X26 cm cage and emerging adults were provided water, powdered sucrose, and powdered egg as a protein source. Adults were egged after 6 days by introducing into the cage a 9 cm petri dish of fresh feces from low-grain fed cattle. The petri dish was removed after sufficient oviposition. After 24 h, first instar larvae were transferred to larger aluminum pans (21 cm square) containing fresh feces. The larvae were allowed to develop in these pans and approximately on the 4th day after transfer, the 3rd instar larvae would begin to wander.

Mineralized granules, used in dissolution studies, were collected from wandering larvae using a procedure developed in our laboratory (A. A. Rueda, 1983, unpublished). Larvae were washed in deionized water and cut into 2-3 pieces which were suspended in 300 um mesh, nylon net in a 600-ml beaker of deionized water with a constant influx of fresh deionized water added dropwise. Groups of 50 - 75 larvae were placed in the net; the granules burst out of the Malpighian tubules and settled to the bottom of the beaker. The constant influx of fresh water insured that lighter tissues and hemolymph did not settle with the granules. After 10 - 15 min, the liquid above the granules was drained leaving the granules in ca. 50 ml of water in the beaker. This suspension was centrifuged and any

visible debris was removed from the granule layer. The granules were rinsed in deionized water, centrifuged, and placed in microbeakers to be used immediately or freeze-dried for storage. From this procedure, 0.5 - 1.0 mg granules/larva could be obtained.

Hemolymph was withdrawn from larvae by puncturing the cuticle laterally and posteriorly to the mouthparts with a 0.15mm diameter minuten pin attached with electric shrink tape to a 5 ul microcapillary tube. The hemolymph flowed directly from the wound into the microcapillary tube.

Stages of development

Throughout this paper, the following stages of development are referred to:

PW = prewandering stage, larvae are in the feeding stages of the 3rd instar

W = wandering stage, a period of ca. 4 h beginning when larvae leave the fecal pat and ending at AR;

AR = anterior retraction, the stage when the larvae first retract into barrel-shaped pupae; mouthparts are still movable;

P = pupal stage, the period beginning at AR and lasting 24 - 30 hr; at this time calcification is first apparent in the cuticle;

A = apolysis, the stage following pupal stage when the puparium separates from the developing pupa within; this occurs ca. 24 hr after W.

Numbers following these stages refer to early, middle, or late time periods within that stage.

Granule dissolution experiments

Granules were treated with buffers of various pH to

determine the effect of pH on granule dissolution. Buffers ranging in pH from 6.3 - 8.2 were prepared from 0.1 N Bis-tris propane (BTP) (Sigma Chemical Company, St. Louis, MO), and 0.1 N HCl. The ionic strength of these buffers was 0.13 - 0.20. Ten ml of buffer were added to centrifuge tubes containing 10 mg of freeze-dried granules. The granules were stirred for 30 sec. After 90 min, the granules were stirred again, centrifuged, and filtered through 0.45 um Gelman filters. The supernatant fluid was analyzed for Ca, P, Mg, and K by plasma emission spectroscopy (KSU Emission Spectroscopy Laboratory). The granules remaining after treatment were prepared for scanning electron microscopy (SEM) by rinsing them with deionized water and allowing them to air dry. They were viewed with an ETEC Autoscan U-1 electron microscope.

In a different dissolution experiment, phosphate buffers of various pH were prepared with 0.1N H_3PO_4 and 0.1N NaOH. Again, 10 mg of granules were weighed into separate centrifuge tubes and treated with 10 ml of buffer for 90 min. The supernatant fluid was discarded and the remaining granules were rinsed and prepared for SEM. The percent of damaged granules at each pH was determined using SEM photographs. Granules with noticeable holes, ridges or dimpling on the surface were counted as damaged.

In a final dissolution experiment, 10 mg of granules were weighed and treated with BTP solutions of different pH values. The granules were stirred for 30 sec, allowed to settle for 30 min, rinsed with deionized water, and centrifuged. One half of the supernatant was withdrawn for

analysis by plasma emission spectroscopy and fresh solution was added to each centrifuge tube. Again, the granules were stirred for 30 sec, allowed to settle for 30 min, and one half of the supernatant fluid was withdrawn. This procedure was repeated three more times. Granules remaining in the centrifuge tubes were prepared for SEM as previously. The weight of granules in the tubes was recalculated as dissolution occurred to account for the concentration of minerals released from the initial weight of granules into the supernatant buffer.

Granules in the meconia and adult Malpighian tubules

Meconia were separated from puparia using fine forceps, were washed in deionized water, and centrifuged. After rinsing, the meconia contents were dropped onto a glass cover slip adhered to an aluminum stub and prepared for SEM. Additional contents were placed on a carbon planchette and used for elemental analysis of individual granules from the meconia using an Ortec Energy Dispersive X-ray Analysis (EDXA) unit attached to the electron microscope.

Ca content in the adult Malpighian tubules was measured using adult flies reared under the usual conditions but supplied with egg protein mixed with powdered milk (3:1). At emergence and every subsequent two days, the Malpighian tubules of 3 female and 3 male flies were dissected, weighed, and homogenized in 0.1N HCl. Ca content was determined by emission spectroscopy as before. Individual granules of the adult tubules were observed by SEM and analyzed by EDXA as

described previously.

pH and osmolality measurements

The pH of the Malpighian tubules was measured with double barrel glass microelectrodes with a tip diameter of 1 um; the pH of the cuticle was measured using a flat-surface microelectrode with a 1 mm sensing diameter (Microelectrodes, Inc., Londonderry, NH). A Corning Model 125 pH meter was used.

The osmolality of the hemolymph was determined on a Wescor Model 5100C vapor pressure osmometer (Wescor, Co., Logan, UT) using 5 ul hemolymph samples.

Mineral budget experiment

Eight separate groups of 100 larvae each were reared in pans containing 1,200 gm feces. Pupae from these groups were placed singly in gelatin capsules. After emergence, the adult fly, first feces, meconium, and puparium from individuals were pooled, ashed, and analyzed for Ca, P, Mg, and K by plasma emission spectroscopy.

Calcium transport experiments

The concentrations of ions in the hemolymph of both face fly larvae and house fly larvae were determined by rearing both flies in the same fecal pat using the procedure of Grodowitz and Broce (1983). Hemolymph samples from individual larvae were pooled and analyzed by plasma emission spectroscopy.

In radioactive tracer experiments, first instar larvae were introduced into radioactive feces prepared by mixing fresh feces with water (50 ml H_2O/550 grams feces) labeled

with 3 uCi Ca45/ml H_2O ($CaCl_2$, New England Nuclear). The final feces label was 1 uCi Ca45/4 gm of liquified feces. Larvae were provided radioactive feces at the rate of 3 gm feces/larva. After 3 days, groups of 50 larvae were transferred from radioactive feces to non-radioactive feces at 10, 7, 6, 4, and 1 h(s) before wandering. These times were approximated according to color and gut contents of the larvae and in relation to the time of first instar larvae transfer. A final group of larvae was allowed to wander from the radioactive feces. Hemolymph samples were collected at several developmental stages and emptied into scintillation vials containing 5 ml of ScintiVerse I (Fisher Scientific Company). Samples were counted on a Beckman LS100 liquid scintillation counter.

Ca45 was also used to determine the amount of Ca present in the Malpighian tubules, cuticle, and remaining tissues through development. First instar larvae were transferred to feces labeled with 1 uCi Ca45/12 gm of feces. Larvae remained in the radioactive feces until wandering. At different stages of pupariation, 3 larvae were dissected in insect Ringer's solution (Barbosa, 1974). The Malpighian tubules, cuticle, and other remaining tissues, rinsed of hemolymph, were dissected and weighed. The tissues were homogenized in 0.1N HCl and centrifuged. An aliquot of the supernatant fluid was counted. The same day, larvae were prepared for total Ca determinations. Whole larvae were homogenized in 0.1N HCl. An aliquot of the supernatant fluid was counted and the

remainder was analyzed for total Ca by atomic absorption (KSU Animal Nutrition Laboratory).

In a final experiment on Ca transport through the hemolymph, two groups of 20 larvae were ligated at the 6th body segment. Twenty larvae were injected with 1 ul 10% isopropyl alcohol and 20 with 1 ul of 10% isopropyl alcohol containing 0.8 ng of ecdysone (Fraenkel and Hsiao, 1967). The microinjection apparatus was that used by Roseland et al. (1983).

RESULTS

Granule Dissolution

The anterior Malpighian tubules of the face fly were greatly distended with mineralized granules during the larval stages. These granules were composed primarily of Ca, P, and Mg with minor amounts of K and trace amounts of Na, Mn, Fe, Zn, and S (Fig. 1). The morphology of granules isolated from the distal portion differed from that of granules isolated from the proximal portion of the Malpighian tubules, but no differences in mineral composition were observed. Granules from the distal portion of the Malpighian tubules were relatively smooth and round (Fig. 2) while those from the proximal portion appeared to be greatly damaged (Fig. 3).

When granules were treated with water of pH 5.0 - 6.0, the pH of the water was increased to a pH of 7.8 - 9.0. The supernatant water contained ca. 10 $\pm$ 2 ppm Ca (mean $\pm$ S.E.) but only 1 $\pm$ 0.5 ppm P. This suggested the pH increase was not due to the release of phosphates from the granules.

The supernatant fluid from granules treated with BTP solutions of pH 6.3 - 8.2 had higher concentrations of Ca, P, and Mg in lower pH solutions (Fig. 4). For example, at pH 6.3 the concentrations of these minerals were: Ca, 12.2; P, 9.7; and Mg, 2.0 ppm; while at pH 8.2 concentrations were 1.1, 1.4, and 0.3 ppm, respectively. The dissolution of the granules can best be described by a decay curve suggesting that as the pH of the bathing fluid decreases the solubility of the granules increases exponentially. K concentrations were not

correlated with pH. The morphology of those granules treated with BTP solutions of pH 7.4 and less was characterized by surface ridges, empty granules with just an outer shell present, and many cracked granules (Fig. 5). The induced damage greatly resembled that observed in granules isolated from the proximal portion of the Malpighian tubules (Fig. 3).

When phosphate buffers were used to treat granules, a greater percentage of damaged granules was observed in lower pH solutions with a maximum of ca. 80% of the granules damaged at a treatment pH of 6.5; 50% of the maximum effect occurred at pH 7.2 near the pKa of the phosphate buffers (Fig. 6). The morphological damage was greatest at pH < 7.4 (Fig. 7 a, b, c, d), slight at pH 7.6 (Fig. 7e) and hardly evident at pH 8.2 (Fig. 7f). Again, the induced damage clearly resembled that observed in granules isolated from the proximal portion of the anterior Malpighian tubules.

When granules were treated with BTP buffers and the supernatant fluid was replaced every 30 min., a significant decrease in the concentrations of Ca, P, Mg, and K/mg of granules treated occurred after the first 30 min. (Fig 8). The solubility of the granules continued to decline in all pH treatments throughout the changes of buffer. The % of the original weight of granules remaining undissolved after each buffer change varied with pH; only ca. 68% of the original weight of granules remained after the last rinse when treated with pH 6.5 buffer compared to ca. 88% of the granules remaining after the last rinse at a treatment pH of 8.0 (Fig. 9).

The ionic strength of the bathing fluid surrounding the granules appeared to only minimally affect granule dissolution (Table 1). Ionic strength did not, therefore, affect any of the in vitro experiments conducted to any considerable degree.

The hindgut contents of larvae throughout the larval-pupal developmental stages were examined by SEM to determine if granules from the Malpighian tubules were emptied into the hindgut. No granules were found in the hindgut from wandering to apolysis. Thus, the site of dissolution could not be the hindgut. However, at a later time in development granules of two types were observed in the meconia. Mineralized granules with the usual composition were observed here, as well as, granules which appeared greatly damaged and which by EDXA analysis contained only large quantities of K (Figs. 10, 11).

Granules were also observed in the contents of the adult Malpighian tubules. However, granules in the adults were often concentrated only in the most distal portion of the tubules, with few or no granules occurring in the middle and proximal portions (Fig. 12). Female flies contained significantly more calcium in the Malpighian tubules than did male flies which completely emptied their tubules shortly after emergence (Fig. 13). No significant increase in calcium content of the adult tubules was observed with age.

pH and Osmolality of Larval Tissues

The pH of the larval Malpighian tubules was found to decrease from the distal to the proximal portion (Table 2). The pH of the hindut was 7.2 - 7.4 while that of the hemolymph

was 7.02 + 0.41. The pH of the cuticle varied with developmental stage (Table 3). At wandering, the pH of the outer cuticle surface was ca. 7.0. By the early pupal stage (P1), the cuticle pH reached its maximum at 8.4; no significant changes in pH occurred after this stage.

The osmolality of the larval - pupal hemolymph was relatively constant through wandering but increased significantly in the late pupal stages (P2, P3) (Fig. 14).

Experiments conducted to determine the distribution of mineral resources stored in the larval stages, revealed that most of the Ca stored in the larvae was later utilized in the puparia. When larvae were reared with 12 gm feces/larva, ca. 70% of the total Ca stored in the larvae was utilized in the puparia, ca. 12 % persisted in the adult flies, and the remainder was excreted as waste (Table 4). The utilization rates of Mg and P were similar to that of Ca; however, less of these minerals were excreted as waste. K was almost equally distributed between the puparia, adult, and waste products.

Calcium Transport to the Puparium

The mineral content of hemolymph from 3rd-instar face fly and house fly larvae raised in the same bovine feces was similar (Table 5). Face fly larvae had slightly higher concentrations of P and Mg ($p < 0.05$) while the concentrations of K and Ca were not significantly different between the two species. The Ca content of face fly hemolymph was measured from the 2nd day of development (2nd instar) to apolysis. Little difference in hemolymph Ca was noted through

development (Fig. 15); these data did not demonstrate that minerals from the Malpighian tubules were being transported, en masse, through the hemolymph. Radioactive tracer experiments studied this process.

Larvae reared in radioactive feces and removed to fresh feces at various times prior to wandering (4, 6, 7, and 10 h) had similar Ca45 increases during the late wandering and anterior retraction stages (W2, W3, AR, Fig 16). It was observed that the initial increase in Ca45 in hemolymph occurred at the same time, irrespective of the time spent in fresh feces. However, Ca45 levels in the hemolymph of larvae removed to fresh feces one hour prior to wandering were similar to levels in larvae maintained in labelled feces until wandering. There were no significant differences in Ca45 activity between pulsed and unpulsed larvae by the late pupal stages or apolysis (P3, A).

The complete transfer of 0.6 - 1.0 mg of Ca from the Malpighian tubules to the cuticle occurred by apolysis (A, Fig. 17). The first significant decrease in Ca content of the Malpighian tubules occurred in the early pupal stage (P1); by apolysis (A) little Ca remained in the tubules. Corresponding increases in Ca content of the cuticle were observed in the same time period. Other tissues, rinsed of hemolymph, included the midgut, hindgut, fat body, and tracheal system and did not show any significant differences in Ca content throughout development (Fig. 17). An approximate estimate of the rate of transport can be determined from the data in

Fig. 17. The average rate of Ca transport from the Malpighian tubules to the cuticle differed with the developmental stages. From W to Ar, a period of ca. 4 h, about 250 ug of Ca were being transported. Assuming the face fly contains a volume of 30 ul of hemolymph, a relatively realistic estimate, ca. 35 ng Ca/min/ul hemolymph were being transported during these early stages. From AR to P1, also ca. 4 h, the estimated transport rate is approximately 31 ng Ca/min/ul hemolymph; by P2 to A, a 12 h period, the estimated rate drastically decreased to 1.2 ng/min/ul hemolymph.

In ligation experiments, larvae injected with 20-OH ecdysone proceeded through anterior retraction and mineralized their puparial cuticle with a visible decrease in the content of the Malpighian tubules. However, control larvae receiving only injections of isopropanol failed to retract and did not show a decrease in the content of the Malpighian tubules.

DISCUSSION

An *in vitro* decrease of the pH of the environment surrounding the granules induced damage similar to that observed in naturally dissolving granules. Granule damage was accompanied by the release of Ca, P, and Mg from the granules; furthermore greater concentrations of supernatant ions were correlated with greater damage. Damaged granules were observed only occasionally in the distal portion of the Malpighian tubules, but they represented the majority of the granules in the proximal portion.

A decrease in pH in the lumen of the Malpighian tubules was observed from the distal to the proximal portions. The pH of the distal portion (8.08) may be the product of buffering by the granules. This buffering may be caused by carbonates in the granules (Darlington, et al. 1983), but was not due to phosphates since they were present only in very small quantities relative to calcium found in supernatant water from granules. The pH of the proximal portion was significantly less (7.35). Though this decrease was not of the magnitude required for maximum dissolution observed *in vitro*, it does greatly increase the rate of dissolution. By extrapolation of the data presented when buffers were continually replaced (Fig. 9), quantities of Ca, P, and Mg, obtained after 1 1/2 h of treatment at pH 6.5, can be obtained after ca. 2 1/2 h at pH 7.3. However, at pH 8.0, similar quantities are not obtained until at least 12 h of treatment.

Thus, the rate of dissolution is nearly 5 times greater at pH 7.3 as that at pH 8.0. The rapid removal of ions from the Malpighian tubules combined with this pH change would seem adequate to bring about dissolution of the mineralized granules.

Darlington et al. (1984) observed that carbonic anhydrase activity increased in the posterior midgut and the hemolymph. These researchers suggested that the enzyme is secreted by the posterior portion of the midgut into the hemolymph where it catalyzes the hydration of CO_2 and the dehydration of HCO_3^- ions, ie., $CO_2 + H_2O \rightleftharpoons HCO_3^- + H_3O^+$. The hydronium ions thus produced would then pass into the proximal portion of the Malpighian tubules and effect dissolution. They further suggested carbonic anhydrase may be involved in a transport system of Ca and HCO_3 ions in the midgut. It is possible carbonic anhydrase mediates a pH change responsible for dissolution, but transport of ions through the gut seems unlikely from our results. Other environmental conditions may affect granule dissolution. Termine and Posner (1970) found that the concentrations of Ca, P, CO_3^{--}, Mg, pyrophosphate, polyphosphates, and macromolecules as well as ionic strength and pH affected the formation of calcium phosphate, <u>in vitro</u>. Ionic strength dces not appear to affect <u>in vitro</u> dissolution of face fly granules appreciably, but any of the other factors, if present in the Malpighian tubules, may affect dissolution.

The pH of the cuticle increased from 7.0 at W to 8.2 - 8.4 by P1. The similarity of this later pH, occurring at the

onset of cuticle mineral deposition, to that found in the distal portion of the Malpighian tubules suggests the face fly is capable of regulating mineralization via pH as it relates to solubility products. This phenomenon has been proposed by Cameron (1985) in the blue crab, Callinectes sapidus (Cameron, 1985) in which calcium carbonate formation depends greatly on pH. Exoskeletal deposits of minerals form readily under alkaline conditions (crab cuticle pH = 8.2), but dissolution occurs if the pH is decreased slightly. This same mechanism may be present in the face fly with pH regulation dependent on bicarbonate buffering. The proposed manner in which the face fly can apparently utilize pH in regulating mineralization is outlined in Fig. 18.

The decreased solubility of granules, in experiments where buffers were continually replaced, suggests decreasing solubility occurred from the outer layers (excluding the cuter covering) to the inner layers. This decrease in solubility may be due to: 1) Decreased permeability of the outer covering, 2) different organic components in the inner vs. the outer layers, or 3) a more compact arrangement of the inner layers allowing less contact with solvent. EDXA of granules did not provide evidence of different mineral components within the different layers. Differences in solubility between granules isolated from different regions of a snail, (Helix), were observed by Simkiss (1976). Different solubilities within the same granules of face fly larvae may also be present. This factor must be overcome by the larvae

dissolution mechanism if the entire mineral resources are to be utilized.

Dissolution studies revealed several components of granule morphology including the concentric nature of the layers of minerals and what appears to be an outer more-insoluble shell or covering surrounding the inner granule layers. This covering may be organic, perhaps membranous, in composition as it was quite insoluble in aqueous buffers; it was frequently observed as the only granule remnant. Figure 19 is a proposed model of granule structure. K^+ ions are shown in close contact with the outer covering; this arrangement would explain the lack of correlation of K with pH dissolution of the granules. Some organic material may serve to structure the inorganic salts in the layers which were observed; and finally the layers are displayed in a more compact arrangement in the more inner granule structure in an attempt to explain decreasing solubility of the inner layers.

Dissolution of the granules may be regulated by the function and development of the Malpighiar tubules. Eastham (1925) noted that peristalsis of the Malpighian tubules brought about the transfer of calcium carbonate from the tubules to the hindgut. In face fly larvae, peristalsis of the gut and tubules may serve as a physical control of granule dissolution allowing the flow of granules from the distal to the proximal portion. In addition, Ryerse (1978, 1980) noted that ecdysone was responsible for the arrest of secretion he observed during the larval-pupal transformation in Calpodes. Mineralization has been linked to ecdysone in the face fly

(Fraenkel and Hsiao, 1967). A repeat of the experiments outlined by Fraenkel and Hsiao (1967) indicated that the usual visible decrease in Malpighian tubule content did not occur in the absence of ecdysone. Since no granules were found in the hindgut throughout pupariation and no Ca45 activity was observed in this tissue, it appears ecdysone may also be responsible for the arrest of secretion to the gut in the face fly. Thus, granule dissolution may occur in the Malpighian tubules as a result of the arrest of secretion to the hindgut and be aided by the physical movement of the Malpighian tubules.

Granules found in the meconia appeared to have been emptied in the late stages of pupariation. Waterhouse, (1950) and Eastham, (1925) observed a similar occurrence in other flies. Those granules which contained large quantities of K may be formed in the posterior Malpighian tubules, hindgut, or midgut and may represent a form of storage excretion for this ion. This type of granule was never observed in the anterior Malpighian tubules.

Granules from adult Malpighian tubules were similar in composition to granules in larvae. No accumulation was documented but data suggested that possible accumulation, followed by excretion or utilization, may occur. The accumulation of granules only in the most distal portion of the tubules in adults, suggests formation was occurring in this segment and/or dissolution was occurring more proximally. Sohal (1977) observed that mineralized 'concretions' did

accumulate in house fly Malpighian tubules with age; thus, it would be expected that the closely related face fly could accumulate granules in the adult Malpighian tubules also. Differences in Ca content between the sexes may be due to differences in diet or may be due to different functions. Taylor (1985) has suggested that mineral reserves in the Malpighian tubules of Calliphora may be utilized in developing ova.

Most of the Ca, P, and Mg stored in the larvae was utilized in the puparia. A slightly greater percentage of Ca was excreted as waste than either of the other ions. However, K was clearly most in excess with nearly one third of the stored mineral excreted in the first adult feces and meconia. This mineral though excreted in large amounts at adult emergence may play a necessary role, such as regulation of ionic transport gradients, for example, in the larval or pupal developmental stages. Larvae reared at a rate of 3 g feces/larva have utilization rates very similar to those of larvae provided 12 g feces/larva. However, less Ca was excreted as waste when less fecal matter was available (unpublished data).

Results of the Ca transport experiments indicated that Ca was transported directly from the Malpighian tubules to the cuticle through the hemolymph. Despite the indication that between 0.6 -1.0 mg of Ca were transported within 24 - 30 h, little increase in hemolymph Ca concentration or osmolality occurred throughout the transport period. Ca transport was first observed in the hemolymph in pulsed larvae at W to AR.

By P1, significantly less Ca was observed in the Malpighian tubules. At this same stage, the first increase in cuticle Ca content was observed. The uptake of Ca in the cuticle may be triggered by the release of Ca from the Malpighian tubules into the hemolymph first observed in tracer studies during W to AR. The fact that Ca45 activity was detected in the hemolymph during the same time period in all pulsed larvae regardless of the number of hours they had been in non-radioactive feces suggests that Ca accumulated last was also utilized last (perhaps in the adult fly or excreted as waste). If this were not so, larvae which were in fresh feces for 10 hr should have shown a delayed increase in Ca45 activity compared to larvae which were pulsed for only 4 hr. This supports the hypothesis that granule formation occurs in the distal portion of the Malpighian tubules with dissolution occurring in the proximal portion.

Great similarity was found in the hemolymph characteristics of face fly larvae and house fly larvae raised in the same environment. Considering the vast differences in the amounts of mineral accumulated between these two species (Grodowitz and Broce, 1983) one would expect their hemolymph to also exhibit these differences. Roseland et al. (1985) found that Ca, P, and Mg were 20 times more prevalent in the puparial cuticle of the face fly as compared to the house fly, yet the hemolymph of the face fly was not much different from that of the house fly. P and Mg concentrations differed somewhat in these two species, suggesting perhaps face flies

have a greater tolerance for higher hemolymph concentrations of these two ions during the larval-pupal transformation. The concentrations of minerals in both species is typical of dipterous larvae as well as other insect species (review by Florkin and Jeuniaux, 1974; Jungreis et al., 1973).

Hemolymph regulation in the face fly larvae, both in terms of mineral content and osmolality, may be due to several factors. Calcium-binding protein activity appears to be of minimal importance (unpublished data). Thus, regulation may be partly due to factors already indicated, such as pH and physical and hormonal factors which control granule dissolution. 20-OH ecdysone may regulate mineralization not only at the Malpighian tubules, as hypothesized, but also at the cuticle. Fraenkel and Hsiao (1967) noted that puparial formation and mineralization of the cuticle does not occur in the absence of ecdysone. Therefore, not only may ecdysone affect dissolution in the Malpighian tubules, but it may also induce events which regulate Ca uptake by the cuticle. In addition, cuticle dehydration initiated at AR and culminating finally in total dehydration following ecdysis, may be important in regulating precipitation of Ca salts at the cuticle which would regulate ionic pools of Ca in this tissue and could further induce Ca transport through the epidermis. Cuticular dehydration is initiated at anterior retraction in a flesh fly, Sarcophaga bullata (Zdarek and Fraenkel, 1972). A diagram of observed and proposed factors affecting the transport processes is shown in Figure 20.

In summary, strong evidence has been presented that a pH

change which occurs from the distal to the proximal portion of the Malpighian tubules is a major effector of granule dissolution and subsequent mineral mobilization. The minerals released from the granules are then apparently transported directly from the proximal portion of the Malpighian tubules to the cuticle, via the hemolymph. Other tissues, including the hindgut and rectum are not apparently involved in calcium transport during this time. Despite the large quantities of minerals being transported, little fluctuation of calcium content or osmolality of the hemolymph is observed. Several regulatory mechanisms that may play a part in this transport system have been suggested. The process of mineralization in the face fly consumes the majority of the minerals stored in the larvae with little Ca, P, or Mg being wasted, but large quantities of K were unutilized. Once adult development has progressed within the pupae, excess minerals are excreted into the meconia or upon emergence by the adult fly. The Malpighian tubules of the adult apparently then regain a similar function as observed in larval Malpighian tubules. A more detailed study of the factors regulating this process of mineralization may lead to a better understanding of mineralization not only in the face fly, but in other organisms as well.

Table 1. Effects of changes in ionic strength on granule solubility.

Ionic strength	Concentration of supernatant ions*			
	P	Mg	Ca	K
0.10	4.66 ± 0.25	1.28 ± 0.13	7.60 ± 0.55	0.18± 0.09
0.20	4.83 ± 0.22	1.55 ± 0.07	8.82 ± 0.82	0.42 ± 0.07
0.30	5.53 ± 0.35	1.77 ± 0.13	10.21 ± 1.40	0.49 ± 0.08
0.50	5.48 ± 0.36	1.68 ± 0.15	9.23 ± 0.36	0.25 ± 0.14

*Mean ± SE (standard error), n = 3.

Table 2. pH of Malpighian tubules, hemolymph, and hindgut.

Tissue	pH
Distal Malpighian tubule	8.08 ± 0.925
Proximal Malpighian tubule	7.35 ± 0.023
Hemolymph	7.02 ± 0.205
Hindgut (by indicators)	7.20-7.40

*Mean pH ± SE (standard error), n = 3.

Table 3. The pH of face fly cuticle through the larval- pupal transformation stages.

Stage	Cuticle pH* Outer surface	Inner surface
W	7.04 ± 0.13	7.20 ± 0.19
AR	7.47 ± 0.12	7.60 ± 0.14
P1	8.42 ± 0.12	7.90 ± 0.20
P2	8.18 ± 0.22	7.80 ± 0.06
A	8.38 ± 0.04	8.43 ± 0.14

Mean pH ± SE (standard error), n = 3-4. Stages are W = wandering, AR = anterior retraction, P1 = early pupal stage, P2 = late pupal stage, A = apolysis.

Table 4. Mineral content (ug) and mean percent of total minerals in tissues of the face fly.

Tissue	P (ug)	%	Mg (ug)	%	Ca (ug)	%	K (ug)	%
First feces	8.23± 2.07	1.55	3.49± 0.78	1.99	59.68± 9.62	7.25	81.48±18.16	31.61
Adult fly	80.44± 7.04	15.09	18.54± 2.07	10.25	95.90± 7.80	11.65	90.95± 6.05	35.30
Meconium	9.83± 1.71	1.85	8.85± 5.18	5.04	37.47±16.40	4.55	9.31± 0.86	3.61
Puparium	432.00±22.62	81.50	145.00± 6.07	82.72	630.11±24.15	76.55	75.94± 8.49	29.48
Total	530.5	99.99	175.88	100.00	823.16	100.00	257.63	100.00

Mineral content (ug) is the mean total mineral ± SE, n = 8.
Percent of total is the percent total mineral represented by the mean.

Table 5. Mineral composition of hemolymph of face fly and house fly larvae raised in the same bovine feces.

Mineral	House fly	Face fly
P	17.21 ± 4.81	34.58 ± 2.53
Mg	13.09 ± 2.01	21.75 ± 1.84
Ca	6.28 ± 1.77	10.49 ± 1.51
K	26.03 ± 2.48	30.25 ± 2.76

Each value is the mean of 6 - 8 determinations ± SE.

Figure 1. Energy-dispersive x-ray analysis trace showing the relative mineral composition of granules isolated from the anterior Malpighian tubules of the face fly.

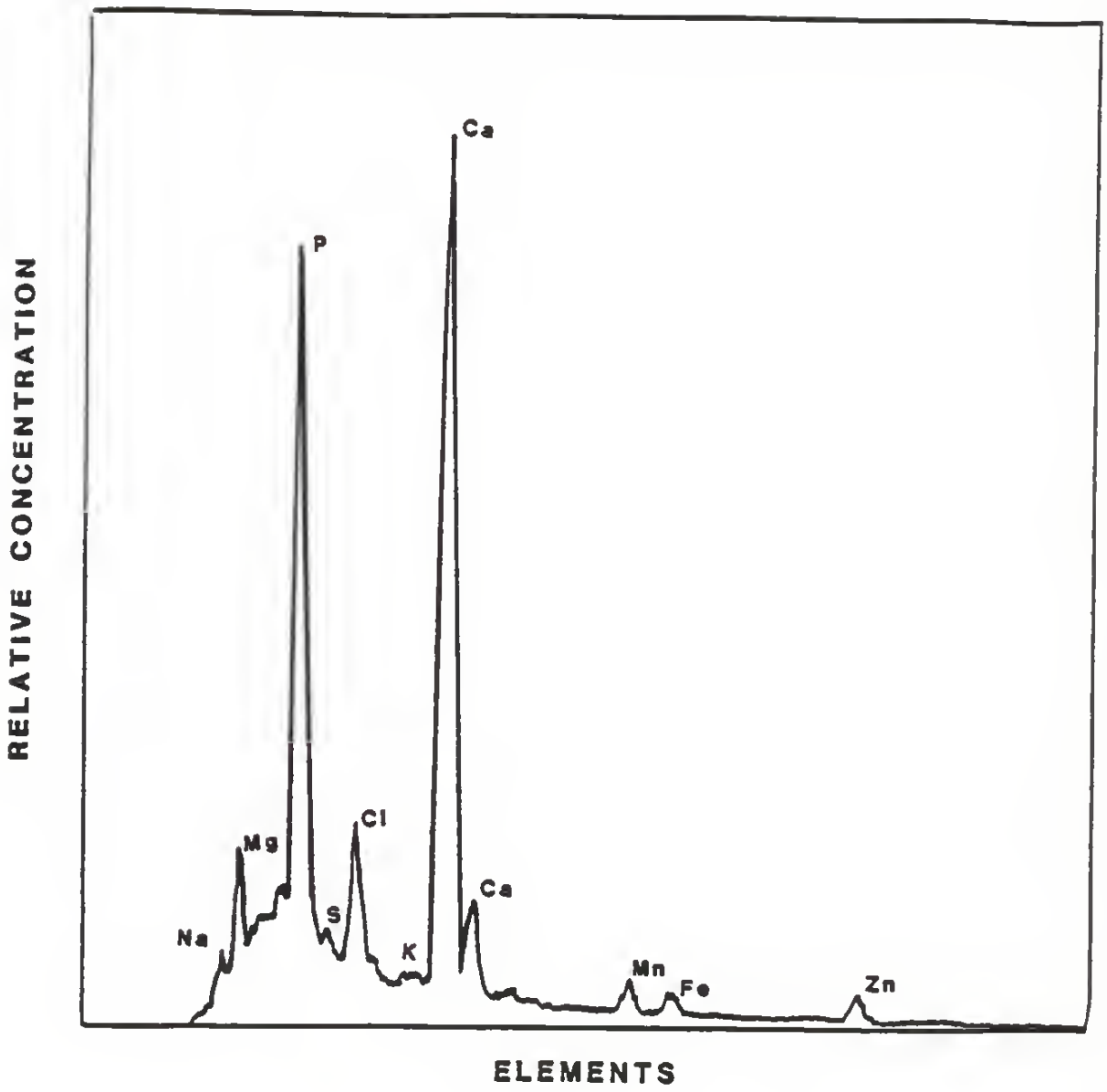

RELATIVE CONCENTRATION
Ca
P
Cl
Mg
Na
S
K
Ca
Mn
Fe
Zn
ELEMENTS

Figure 2. SEM image of mineralized granules from the distal portion of the anterior Malpighian tubules of the face fly.

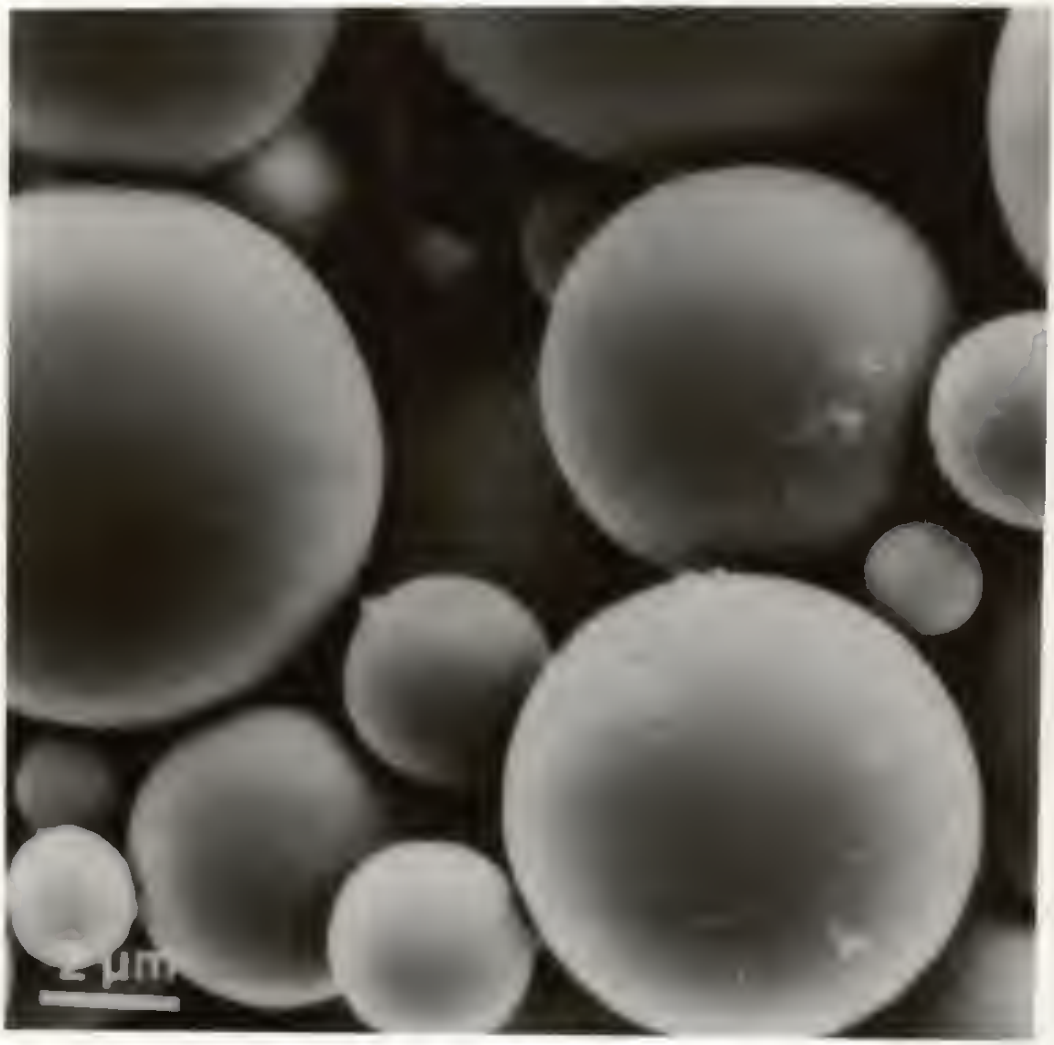

2 µm

Figure 3. SEM image of mineralized granules from the proximal portion of the anterior Malpighian tubules of the face fly.

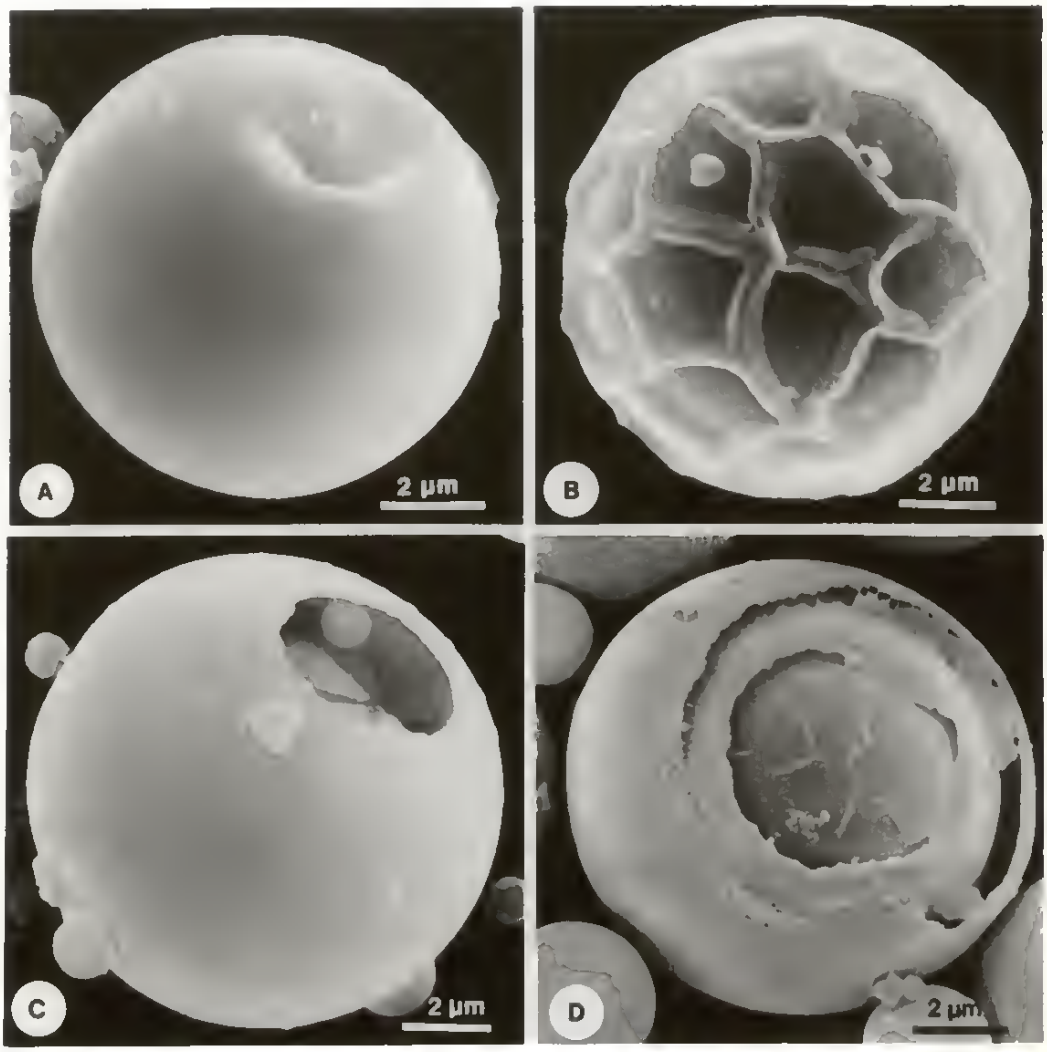

A
2 µm
B
2 µm
C
2 µm
D
2 µm

Figure 4. Dissolution of face fly granules isolated from the anterior Malpighian tubules: Supernatant concentrations of Ca, Mg, P, and K in bis-tris propane buffered solutions as a function of pH. $\bar{X} \pm SE$, n = 3.

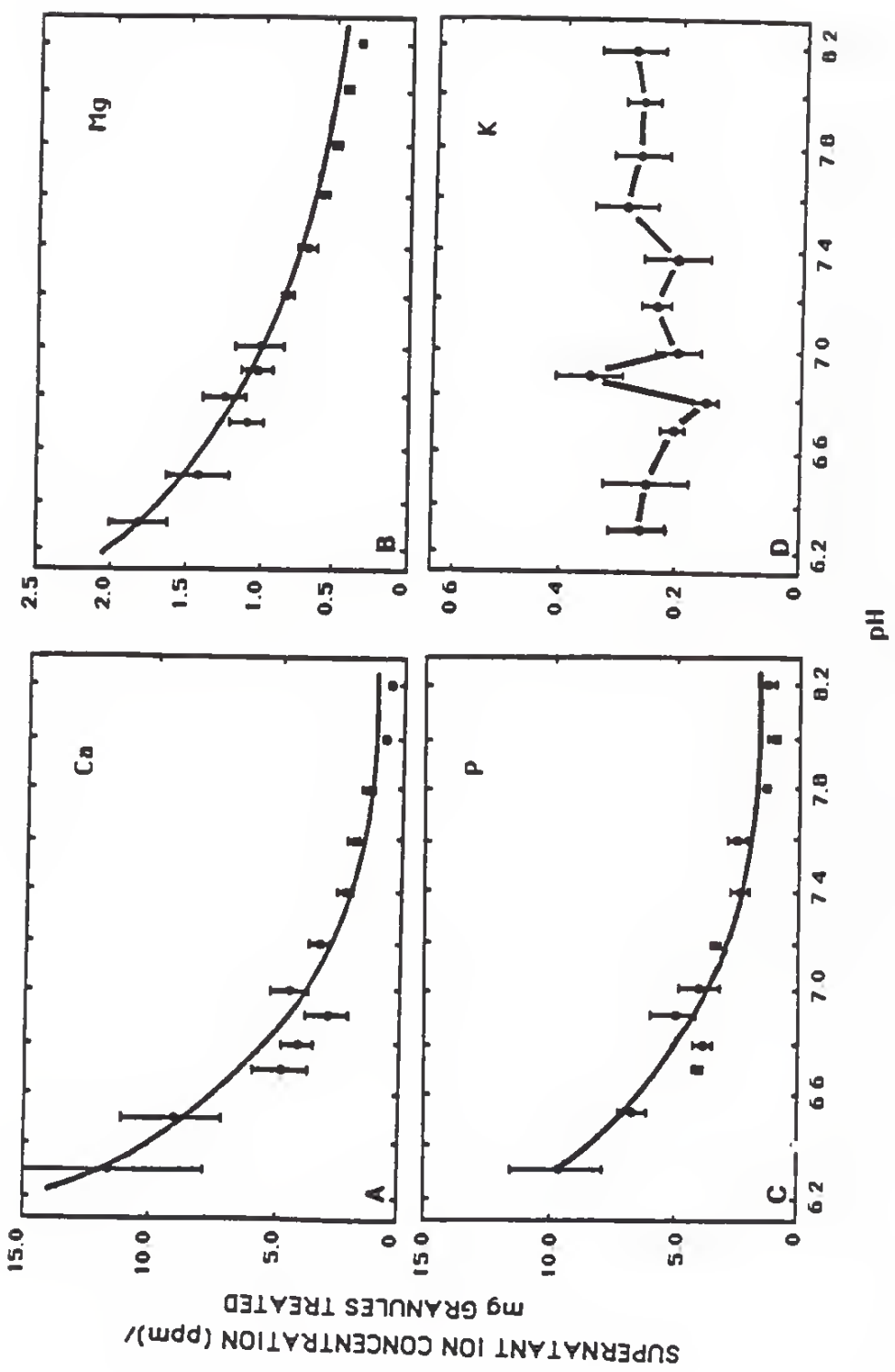

Ca
Mg
P
K
A
B
C
D
15.0
10.0
5.0
0
2.5
2.0
1.5
1.0
0.5
0.6
0.4
0.2
6.2
6.6
7.0
7.4
7.8
8.2
pH
SUPERNATANT ION CONCENTRATION (ppm)/
mg GRANULES TREATED

Figure 5. Morphology of granules treated with Bis-tris propane buffers of pH 7.4 and less. Note the outer shell remaining in a) and b), the collapsing outer layer in c) and d), and the cracked granules in e) and f).

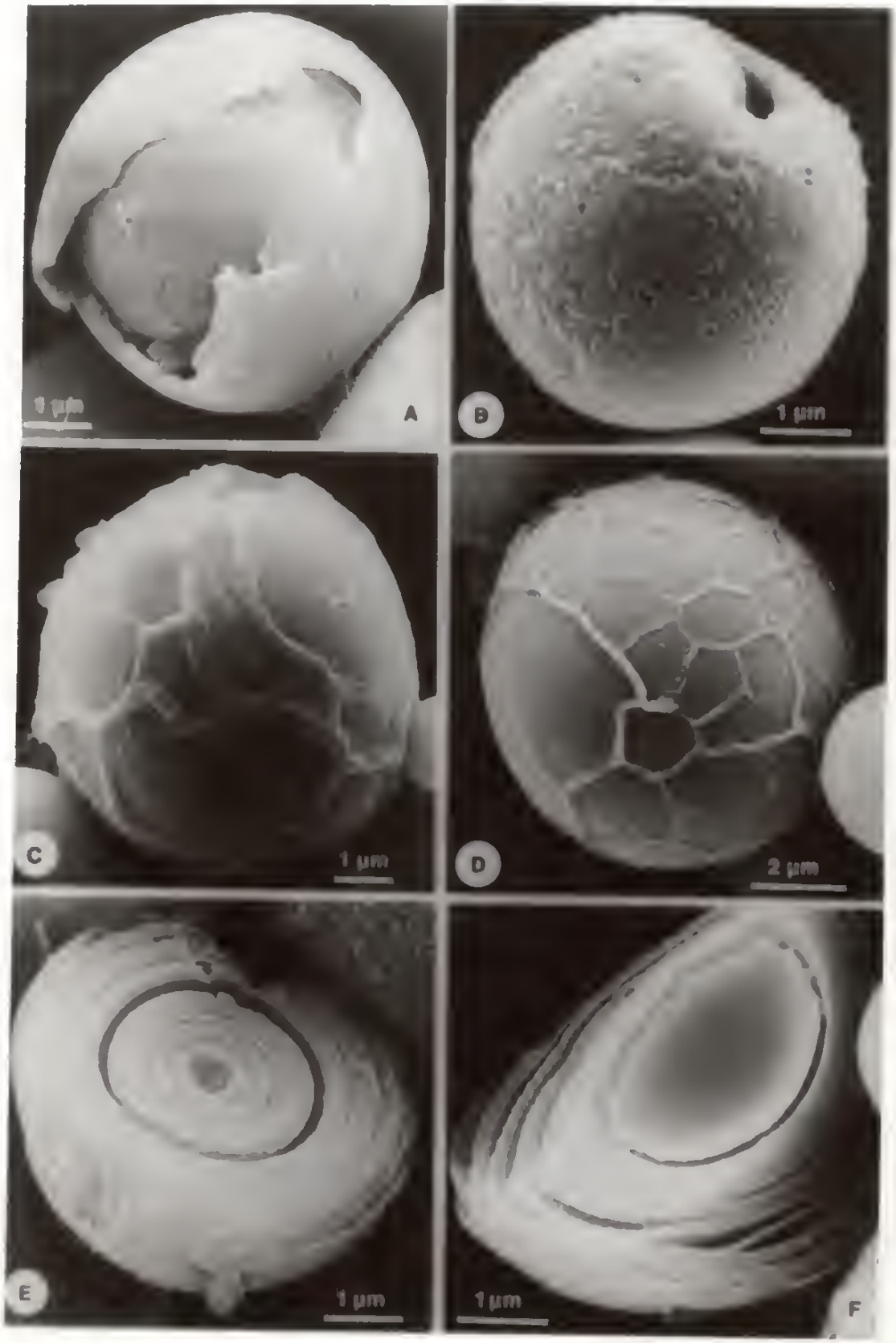

A
1 µm
B
1 µm
C
1 µm
D
2 µm
E
1 µm
F
1 µm

Figure 6. The percentage of face fly granules showing signs of morphological damage in phosphate buffers of different pH. $\bar{X} \pm SE$, $n = 3$.

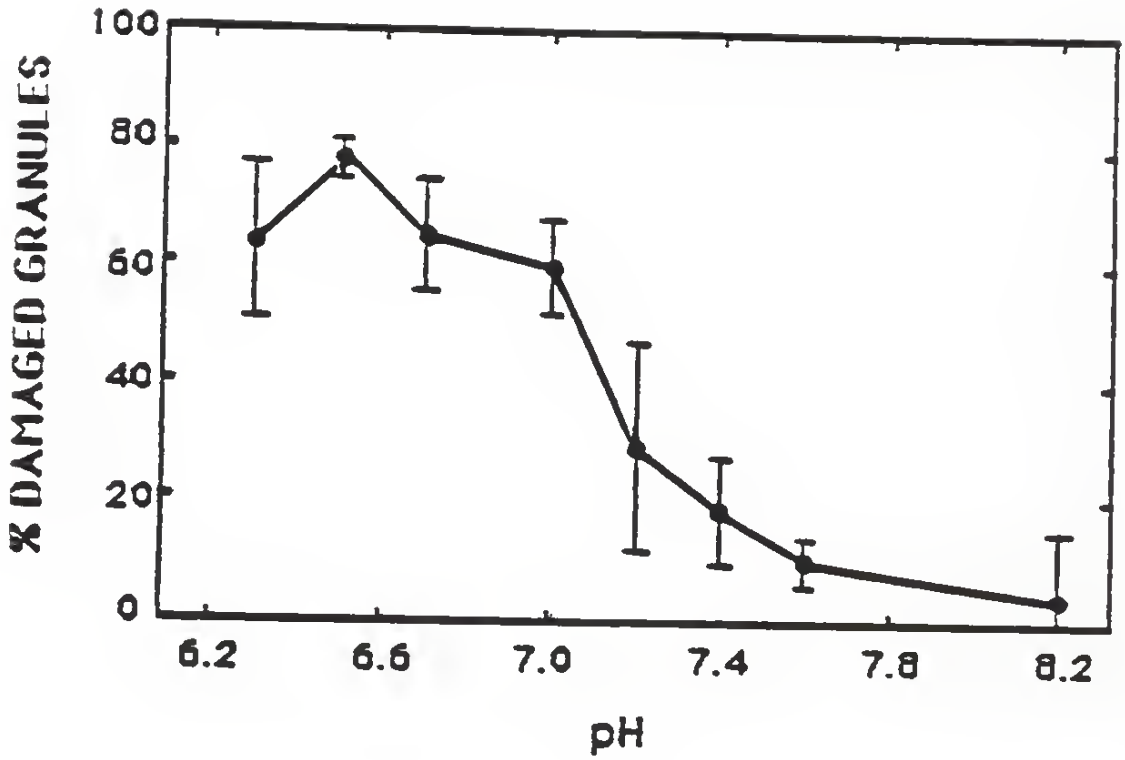

% DAMAGED GRANULES
100
80
60
40
20
0
6.2
6.6
7.0
7.4
7.8
8.2
pH

Figure 7. Morphology of face fly granules treated in phosphate buffers of various pH. Granule damage was great at pH 7.4 and less (a, b, c, d), slight at pH 7.6 (e) and hardly evident at pH 8.2 (f).

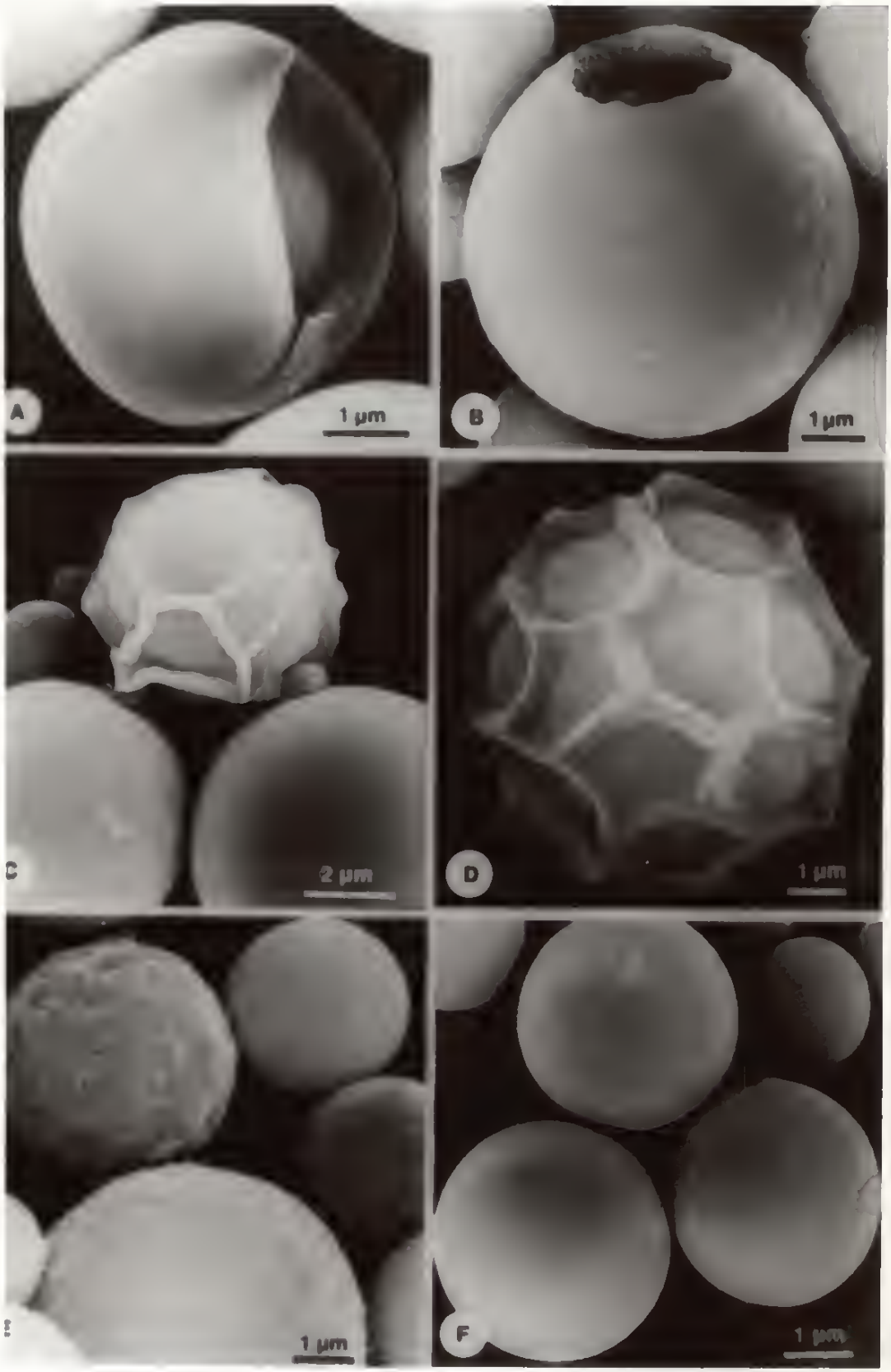

A
1 µm
B
1 µm
C
2 µm
D
1 µm
1 µm
F
1 µm

Figure 8. The supernatant concentration of Ca, P, K, and Mg in Bis-tris propane treatments of face fly granules when buffers were changed at 30-min. intervals. n = 3.

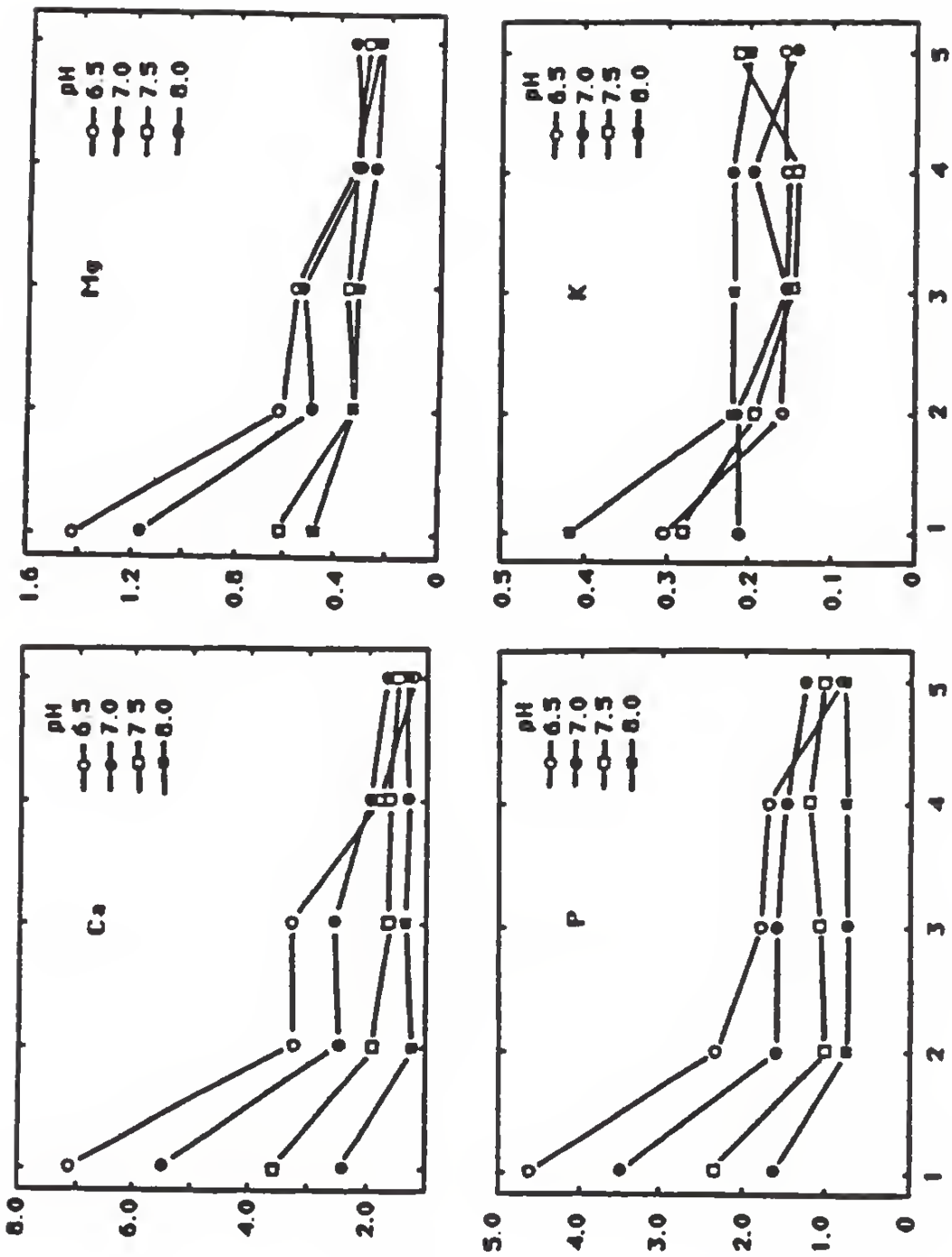

pH
6.5
7.0
7.5
8.0
Mg
K
Ca
P
1.6
1.2
0.8
0.4
0
0.5
0.4
0.3
0.2
0.1
8.0
6.0
4.0
2.0
5.0
4.0
3.0
2.0
1.0
1
2
3
4
5
BUFFER CHANGE NO.
ION CONCENTRATION (ppm) / mg GRANULES

Figure 9. Calculated weight of granules remaining after each change of buffer shown in Figure 8 in 4 pH treatments. n = 3.

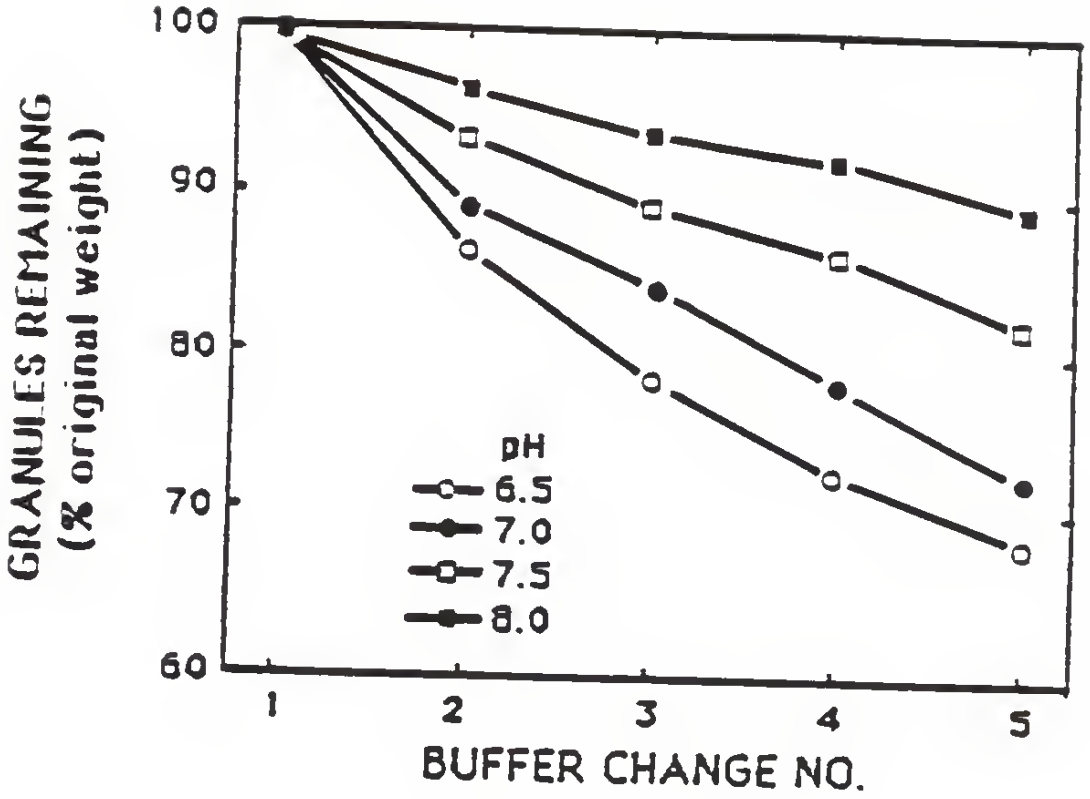

GRANULES REMAINING
(% original weight)
100
90
80
70
60
1
2
3
4
5
BUFFER CHANGE NO.
pH
6.5
7.0
7.5
8.0

Figure 10. Energy-dispersive x-ray analysis trace showing the mineral composition of granules with signs of severe damage when isolated from face fly meconia. The large peak of K alone clearly differentiates these granules from the mineralized granules of the anterior Malpighian tubules (compare with Fig. 1).

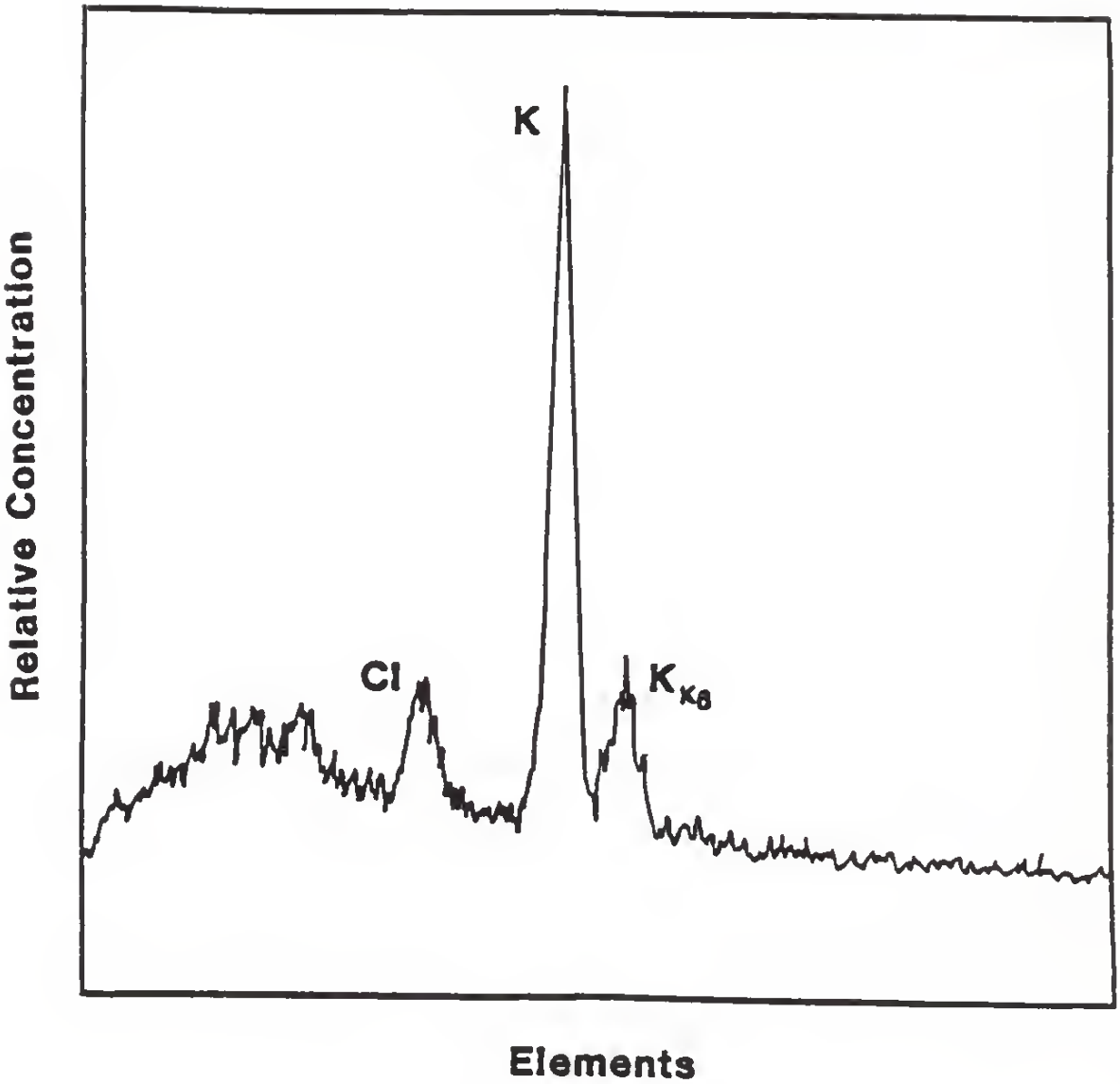

K
Cl
$K_{K\beta}$
Relative Concentration
Elements

Figure 11. Morphology of granules isolated from the face fly meconia.

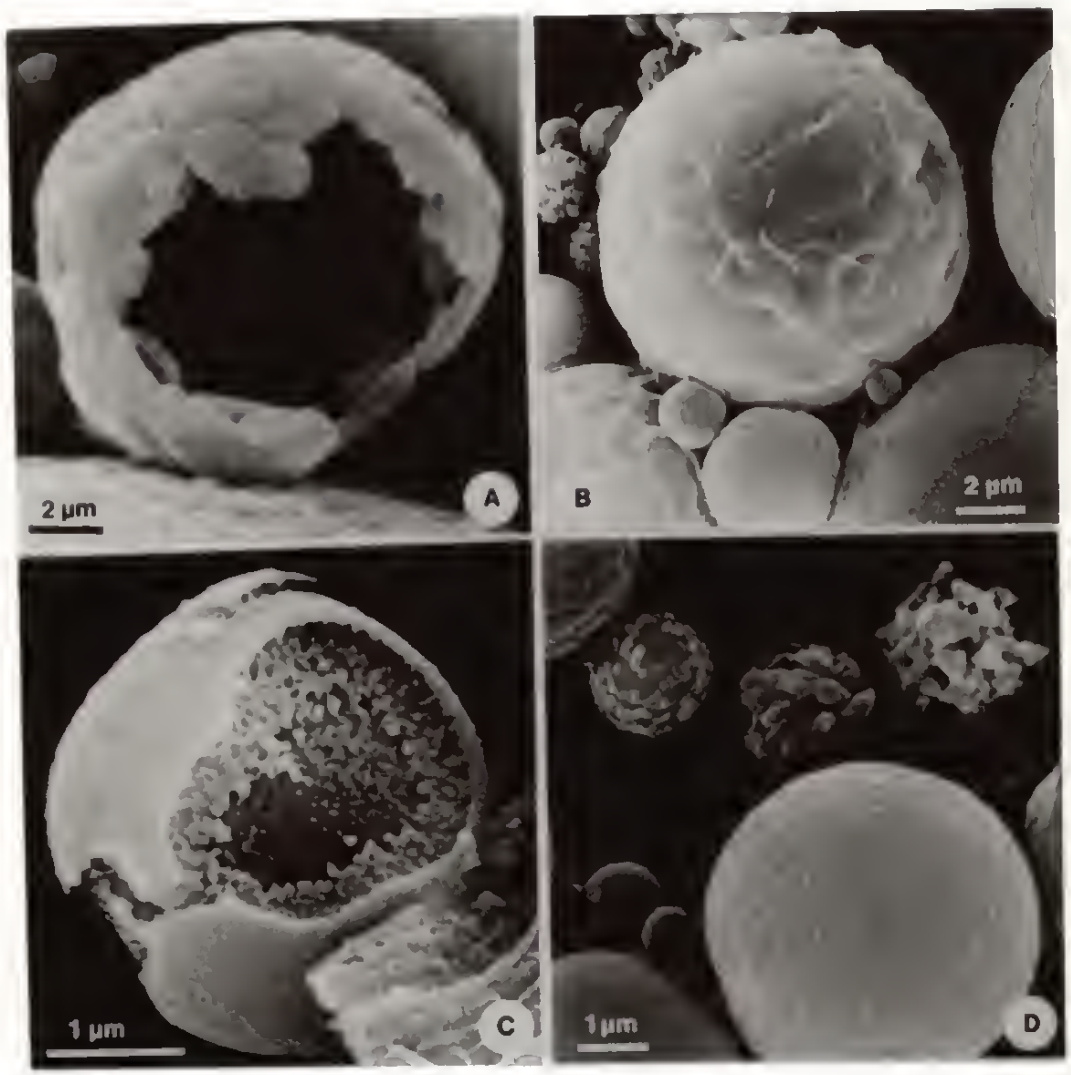

2 µm
A
B
2 µm
1 µm
C
1 µm
D

Figure 12. Diagram of the face fly adult Malpighian tubules showing the concentration of granules only in the most distal portion of the anterior Malpighian tubules.

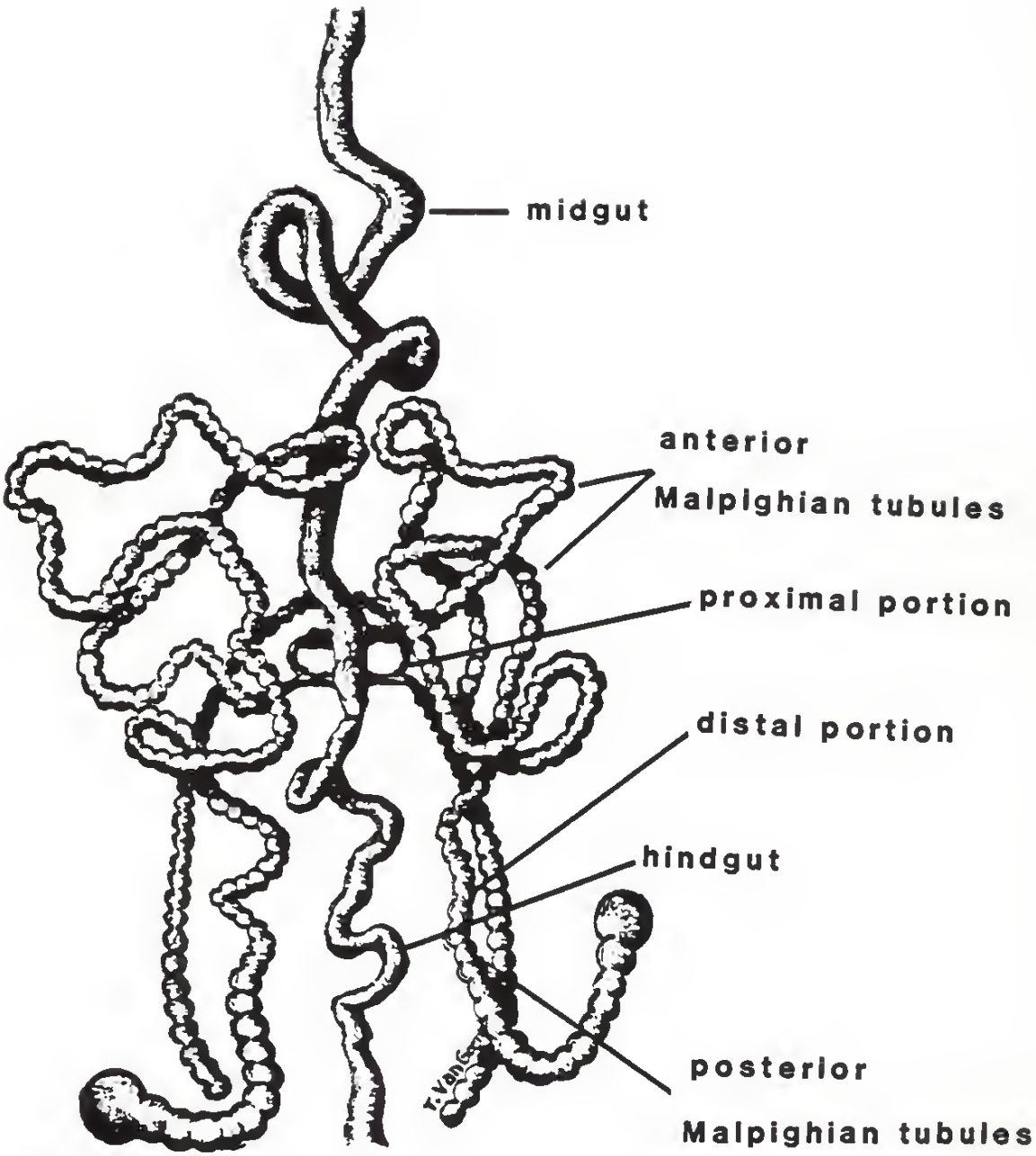

midgut
anterior
Malpighian tubules
proximal portion
distal portion
hindgut
posterior
Malpighian tubules

Figure 13. The total calcium/g wet tissue in the anterior Malpighian tubules of both male and female face flies as a function of age (days post-emergence). $\bar{X} \pm SE$, n = 3.

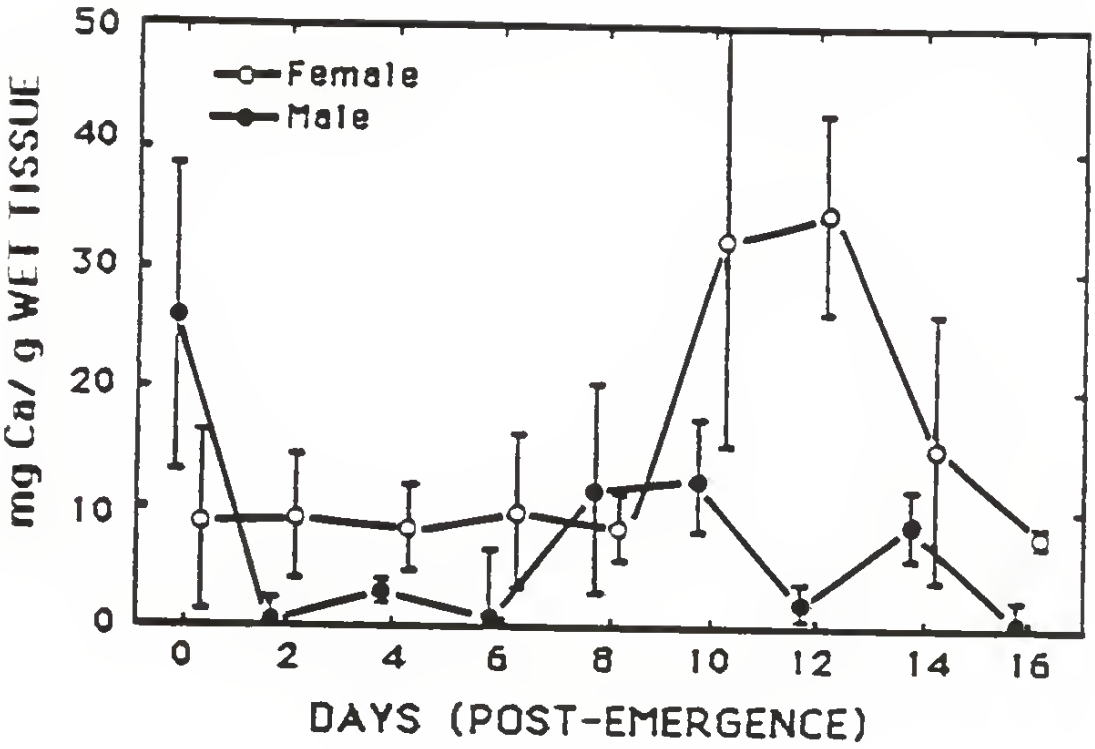

50
40
30
20
10
0
mg Ca/ g WET TISSUE
Female
Male
0
2
4
6
8
10
12
14
16
DAYS (POST-EMERGENCE)

Figure 14. The osmolality of face fly hemolymph during the larval-pupal transformation stages. PW1 = early prewandering, PW2 = late prewandering, ca. 12 hours before wandering, W1 = early wandering, W2 = late wandering, AR = anterior retraction, P1 = early pupal, P2 = mid pupal, and P3 = late pupal stage, A = apolysis. $\bar{X} \pm SE$, n = 3.

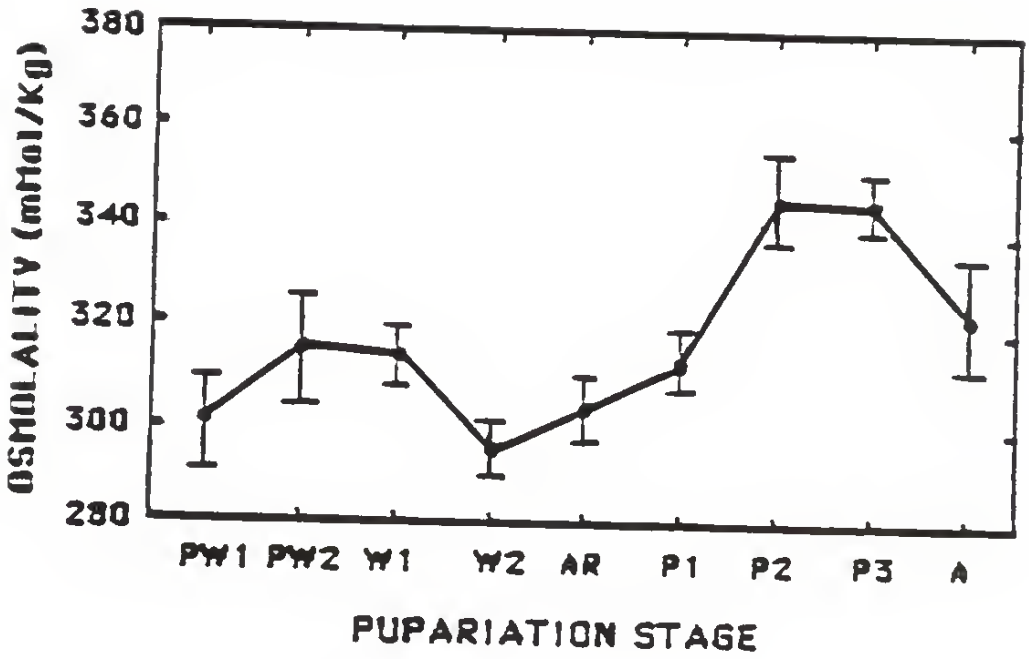

OSMOLALITY (mMol/Kg)
380
360
340
320
300
280
PW1
PW2
W1
W2
AR
P1
P2
P3
A
PUPARIATION STAGE

Figure 15. The concentration of Ca (meq/l) in face fly hemolymph through larval-pupal development. D2 = day 2, 2nd instar period, D3 = day 3, late 2nd to early 3rd instar, PW1 = early prewandering, PW2 = late prewandering, W1 = early wandering, W2 = late wandering, AR = anterior retraction, P1 = early pupal stage, and A = apolysis. $\bar{X} \pm SE$, n = 3 - 4.

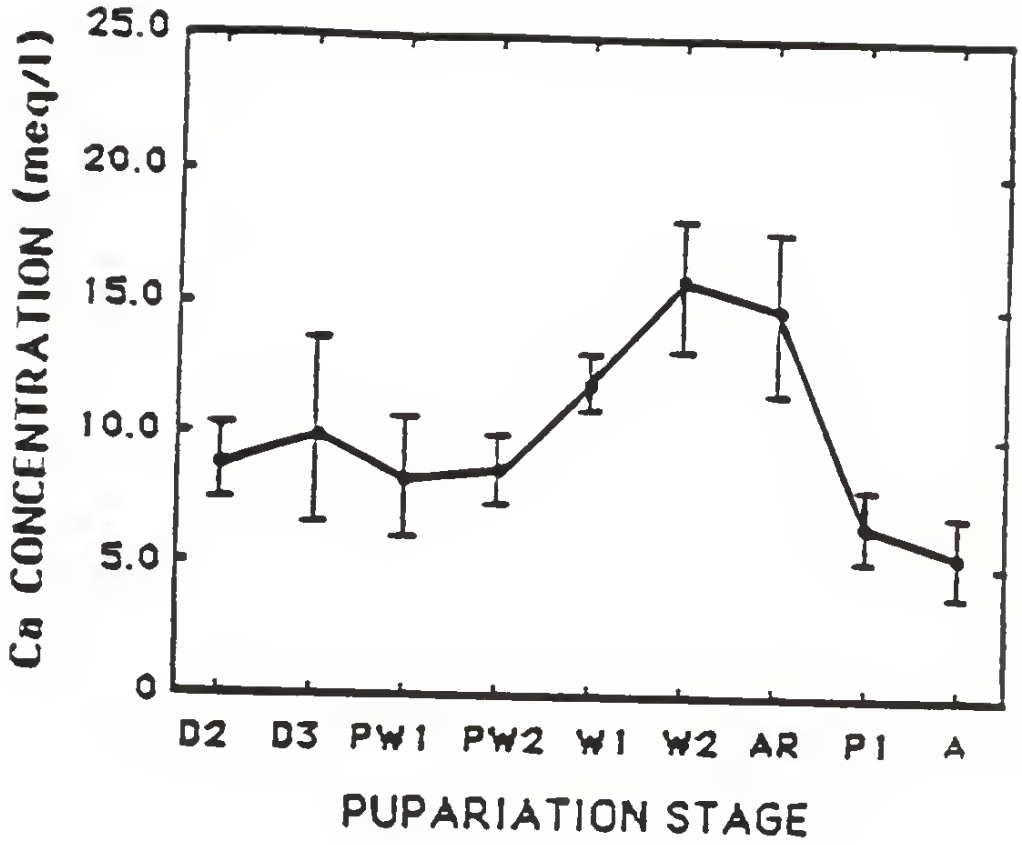

Ca CONCENTRATION (meq/l)
25.0
20.0
15.0
10.0
5.0
0
D2
D3
PW1
PW2
W1
W2
AR
P1
A
PUPARIATION STAGE

Figure 16. Ca45 activity ($\bar{X}$cpm/ul ± SE) of hemolymph of face fly larvae grown continuously in radioactive feces (hours pulsed = 0) and when larvae were pulsed for variable lengths of time prior to wandering to non-radioactive feces (ie, 10, 7, 6, 4 and 1 hr(s)). n = 5. PW1 = early prewandering stage, W = wandering, AR = anterior retraction, P1 = early pupal stage, P2 = late pupal stage, and A = apolysis. n = 3 - 5.

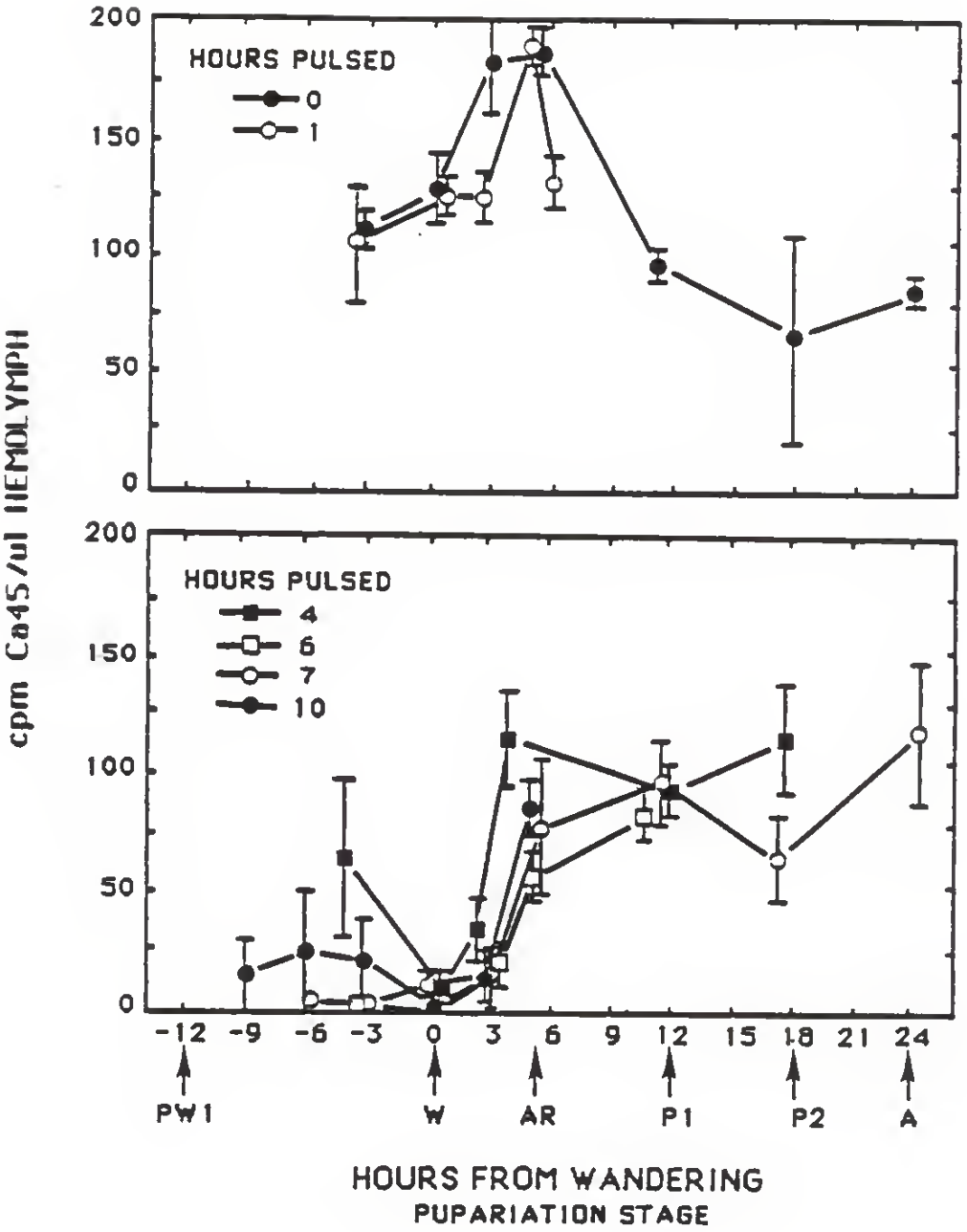

HOURS PULSED
0
1
HOURS PULSED
4
6
7
10
200
150
100
50
0
cpm Ca45/ul HEMOLYMPH
-12 -9 -6 -3 0 3 6 9 12 15 18 21 24
PW1
W
AR
P1
P2
A
HOURS FROM WANDERING
PUPARIATION STAGE

Figure 17. Total Ca (ug) of the Malpighian tubules, cuticle, and other tissues during the larval-pupal transformation. "Other tissues" included the gut, fat body, and tracheal system and were rinsed of hemolymph. W = wandering, AR = anterior retraction, P1 = early pupal, P2 = late pupal stages, and A = apolysis. $\bar{X} \pm SE$, n = 3.

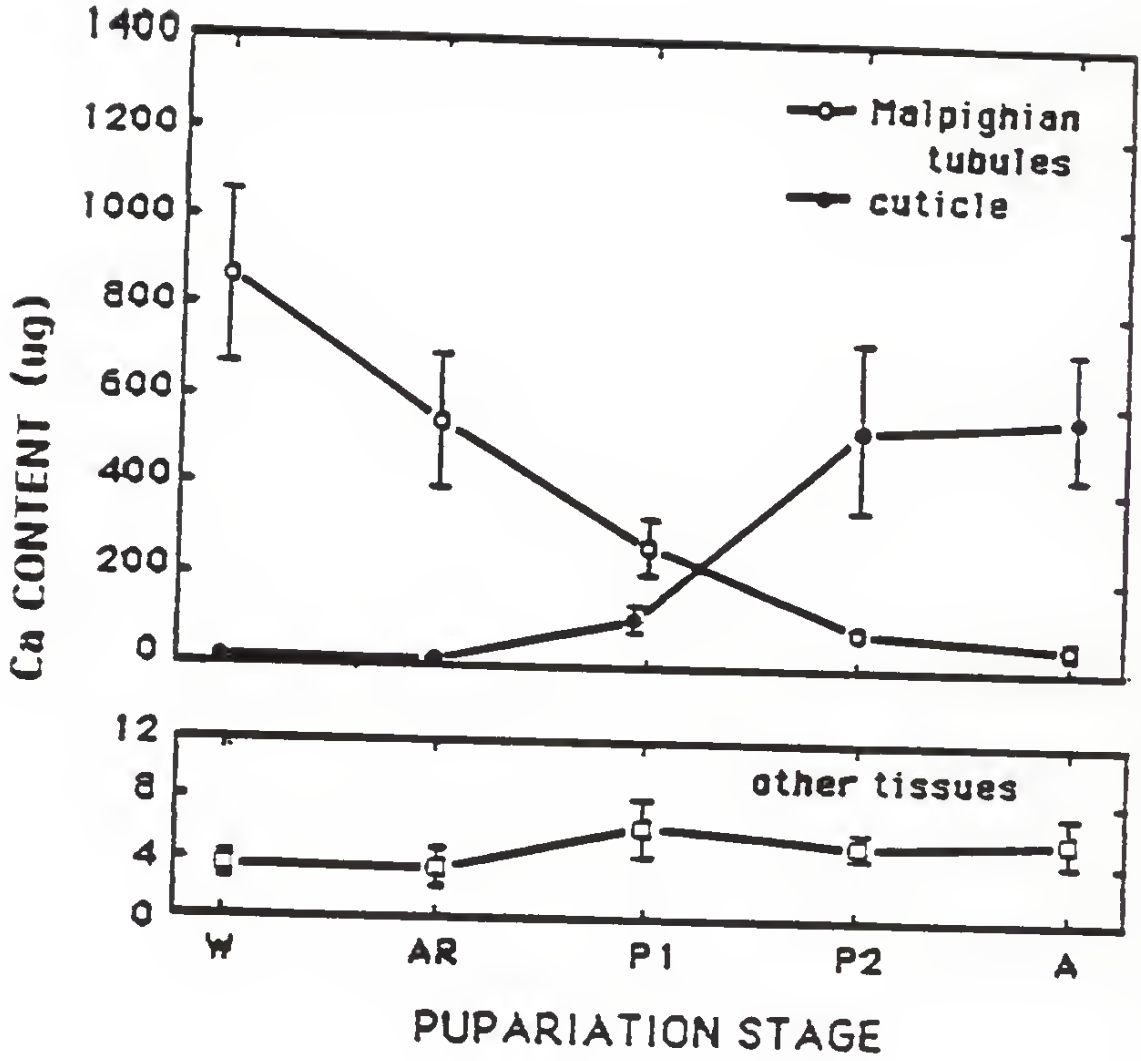

1400
1200
1000
800
600
400
200
0
Ca CONTENT (ug)
Malpighian tubules
cuticle
12
8
4
0
other tissues
W
AR
P1
P2
A
PUPARIATION STAGE

Figure 18. A hypothetical scheme showing the proposed role of pH in formation and dissolution of face fly granules in the Malpighian tubules and the deposition of minerals in the cuticle.

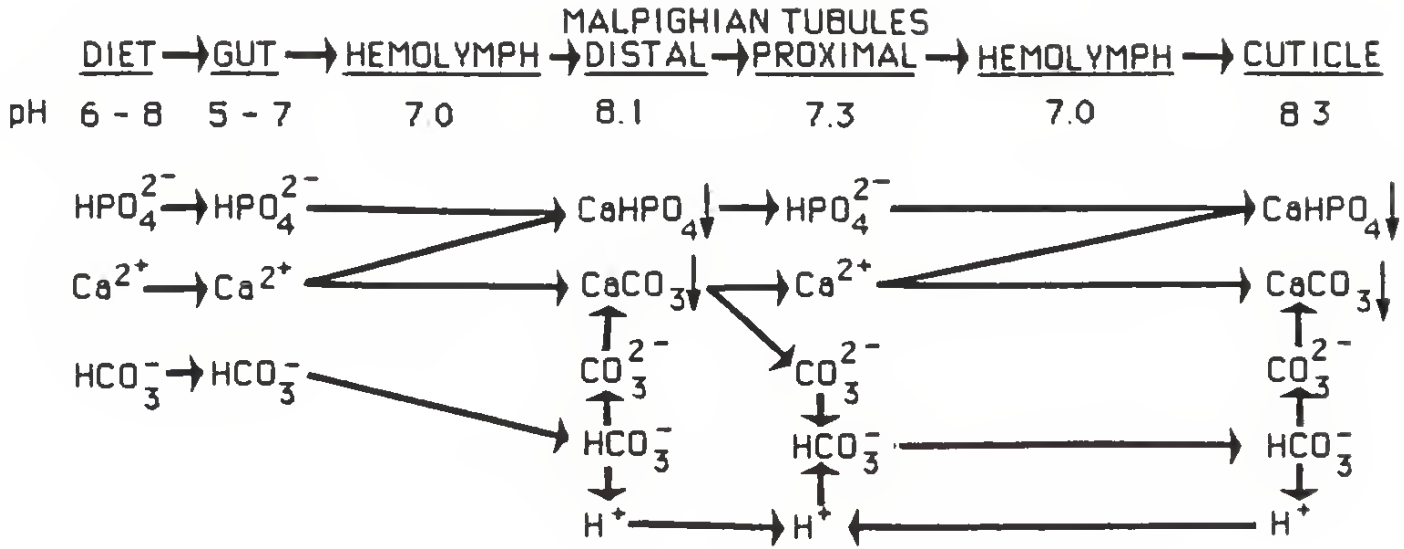

MALPIGHIAN TUBULES
DIET
GUT
HEMOLYMPH
DISTAL
PROXIMAL
HEMOLYMPH
CUTICLE
pH
6 - 8
5 - 7
7.0
8.1
7.3
7.0
8 3
HPO_4^{2-}
Ca^{2+}
HCO_3^-
$CaHPO_4$
$CaCO_3$
CO_3^{2-}
H^+

Figure 19. The proposed structure of mineralized granules isolated from face fly Malpighian tubules. Structure derived from observations and data obtained from dissolution studies.

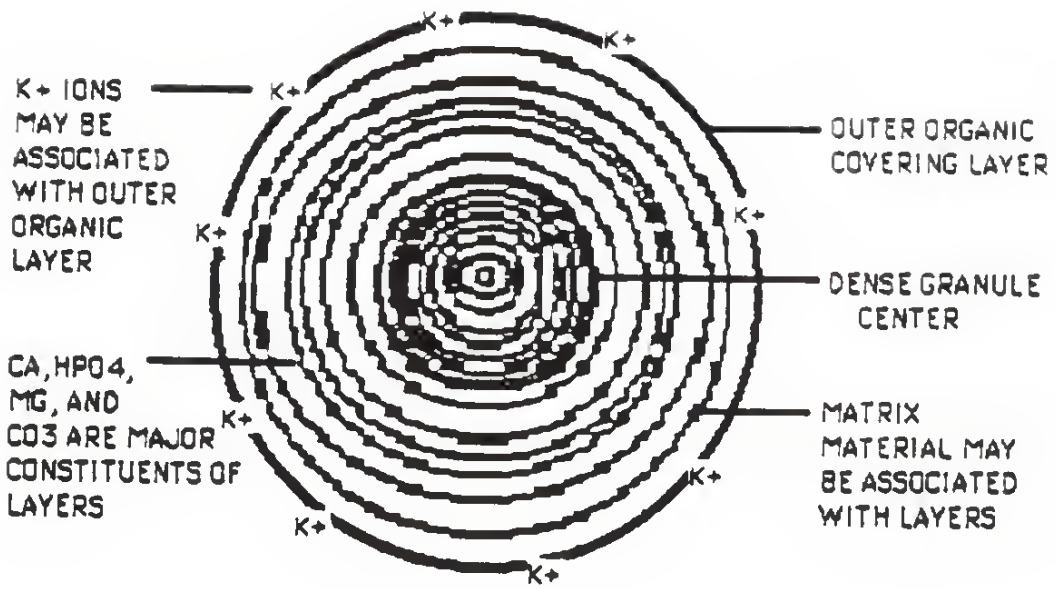
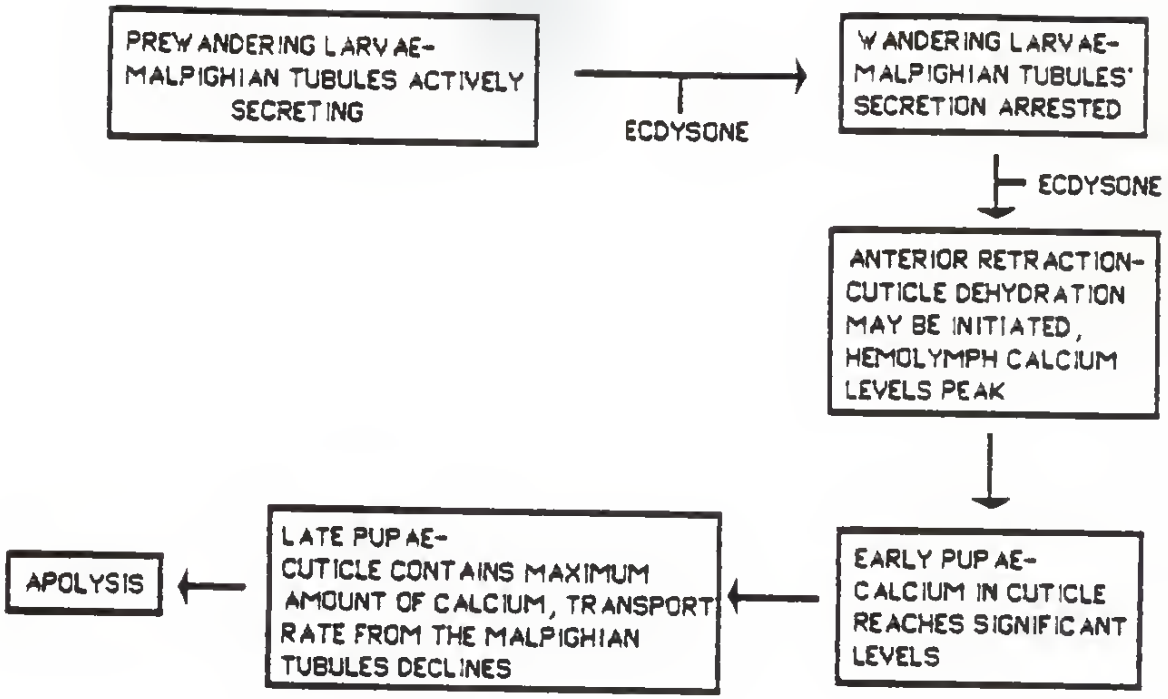

K+ IONS MAY BE ASSOCIATED WITH OUTER ORGANIC LAYER
CA, HPO4, MG, AND CO3 ARE MAJOR CONSTITUENTS OF LAYERS
OUTER ORGANIC COVERING LAYER
DENSE GRANULE CENTER
MATRIX MATERIAL MAY BE ASSOCIATED WITH LAYERS
K+

Figure 20. Some factors other than pH which may play a role in dissolution of granules and subsequent mineral transport.


PREWANDERING LARVAE-
MALPIGHIAN TUBULES ACTIVELY
SECRETING
ECDYSONE
WANDERING LARVAE-
MALPIGHIAN TUBULES'
SECRETION ARRESTED
ECDYSONE
ANTERIOR RETRACTION-
CUTICLE DEHYDRATION
MAY BE INITIATED,
HEMOLYMPH CALCIUM
LEVELS PEAK
EARLY PUPAE-
CALCIUM IN CUTICLE
REACHES SIGNIFICANT
LEVELS
LATE PUPAE-
CUTICLE CONTAINS MAXIMUM
AMOUNT OF CALCIUM, TRANSPORT
RATE FROM THE MALPIGHIAN
TUBULES DECLINES
APOLYSIS

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APPENDIX

APPENDIX

The effects of various ions on the dissolution properties of the granules were measured by dialysis at 4° C using Ca45 labelled granules and BTP buffer. The treatment of granules with 0.005 - 0.10 N $CaCl_2$ in BTP caused a decrease in granule solubility (Fig.1). Phosphate and pyrophosphate ions may enhance granule dissolution. The mean Ca45 levels in these treatments was greater than any other treatment observed. These data suggest the removal of Ca from the vicinity of the granules is important in the granule dissolution mechanism within face fly larvae while the presence of phosphate may slightly enhance the dissolution mechanism.

Granules were also treated in 2 other buffers besides BTP and phosphate buffer and the concentration of Ca/mg granules treated was measured. Ca was analyzed by atomic absorption. (N-(2-Acetamido)-2-iminodiacetic acid (ADA) and triethanolamine (TEA) (Sigma Chemical Co., St. Louis, MO) were used. The dissolution of granules in these buffers was similar to that observed when granules were treated with BTP (Table 1). The amorphous nature of the calcium and phosphate in the mineralized granules increases the solubility ca. 10 times compared to the standard crystalline $CaHPO_4$ (Fig. 2).

Larvae were raised in 3 g feces/larvae and the percent utilization of Ca in the puparia, adult fly, and the percent excreted as waste was calculated. The utilization rate is similar to that seen when larvae were provided 12 g feces/larva. However, much less Ca was found in the waste

products (Table 2). When larvae were provided 12 g feces/larva waste accounted for ca. 12 % of the total Ca (see Results section); with 3 g feces/larvae, only 1% of the Ca was excreted as waste.

Table 3 illustrates the effect of pulsing larvae in the middle 3rd instar to fresh feces and then returning them to the labelled feces within 12 h. The Ca45 activity profile was similar to that observed in larvae that were not pulsed. It was not possible to detect the flux of unlabelled Ca into the hemolymph.

Finally, an experiment was conducted to determine if Ca binding proteins may be present in the hemolymph to aid regulation of the osmolality and concentration of Ca during the transport process. The method used to determine binding was micropartition filtration (Amicon Co., Danvers, MA). This method involved the centrifugation of hemolymph through a molecular weight cutoff membrane having a cutoff of 5000 D. The results as shown in Table 4 indicate a small proportion of Ca could be bound to a hemolymph protein; however, a better system for determining calcium binding is needed and results may be overestimated.

Figure 1. The effects of various ions on granule solubility in Bis-tris propane buffer. The dissolution curve represents the Ca45 cpm measured when no additional ions are present. Each point in the treatment groups is the mean of 3 determinations ± SE.

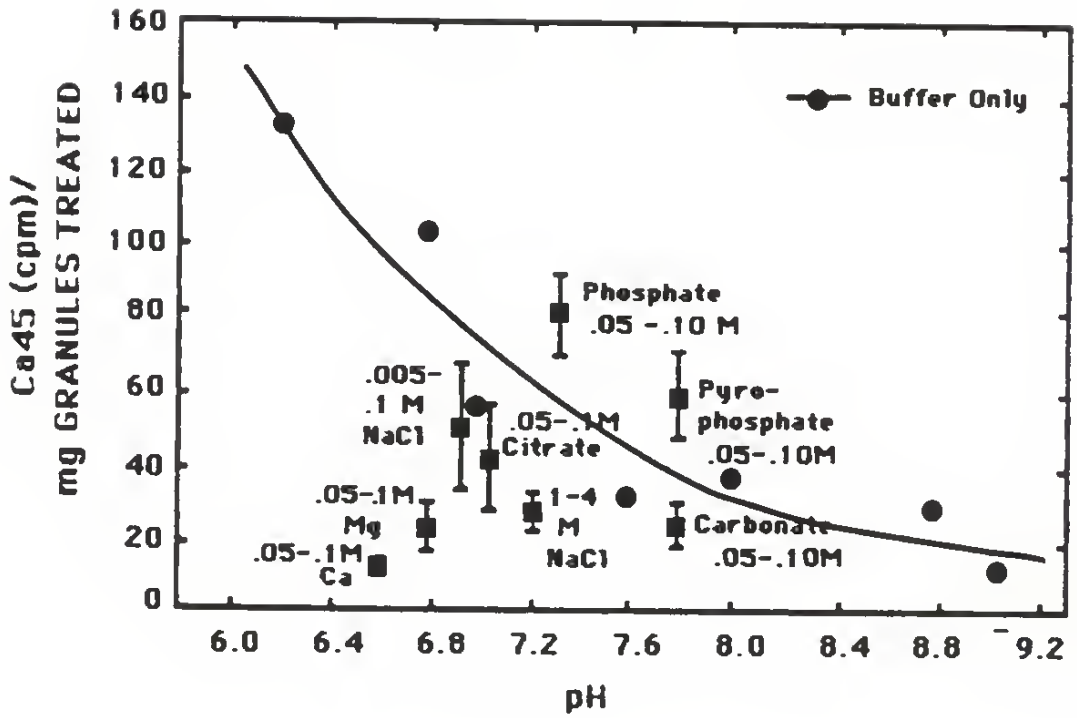
Ca45 (cpm)/
mg GRANULES TREATED
160
140
120
100
80
60
40
20
0
6.0
6.4
6.8
7.2
7.6
8.0
8.4
8.8
9.2
pH
Buffer Only
Phosphate
.05 -.10 M
.005-
.1 M
NaCl
.05-.1M
Citrate
Pyro-
phosphate
.05-.10M
.05-.1M
Mg
.05-.1M
Ca
1-4
M
NaCl
Carbonate
.05-.10M

Table 1. Dissolution of mineralized granules in two buffers, ADA pH 6.3 - 7.0, and TEA pH 7.20 - 8.20.

pH	ppm Ca/mg granules ± 2SE*
6.3	26.80 ± 1.47
6.5	24.51 ± 1.49
6.7	26.10 ± 1.48
6.8	26.73 ± 2.71
6.9	20.41 ± 0.47
7.0	18.07 ± 5.06
7.2	4.65 ± 0.96
7.4	3.94 ± 0.58
7.6	3.48 ± 0.36
7.8	3.04 ± 0.57
8.0	2.63 ± 0.10
8.2	1.80 ± 0.18

*Values are mean supernatant Ca concentrations ± 2SE (standard error), n=3.

Figure 2. The comparative dissolution of mineralized granules isolated from the face fly Malpighian tubules (A) and standard $CaHPO_4$ (B).

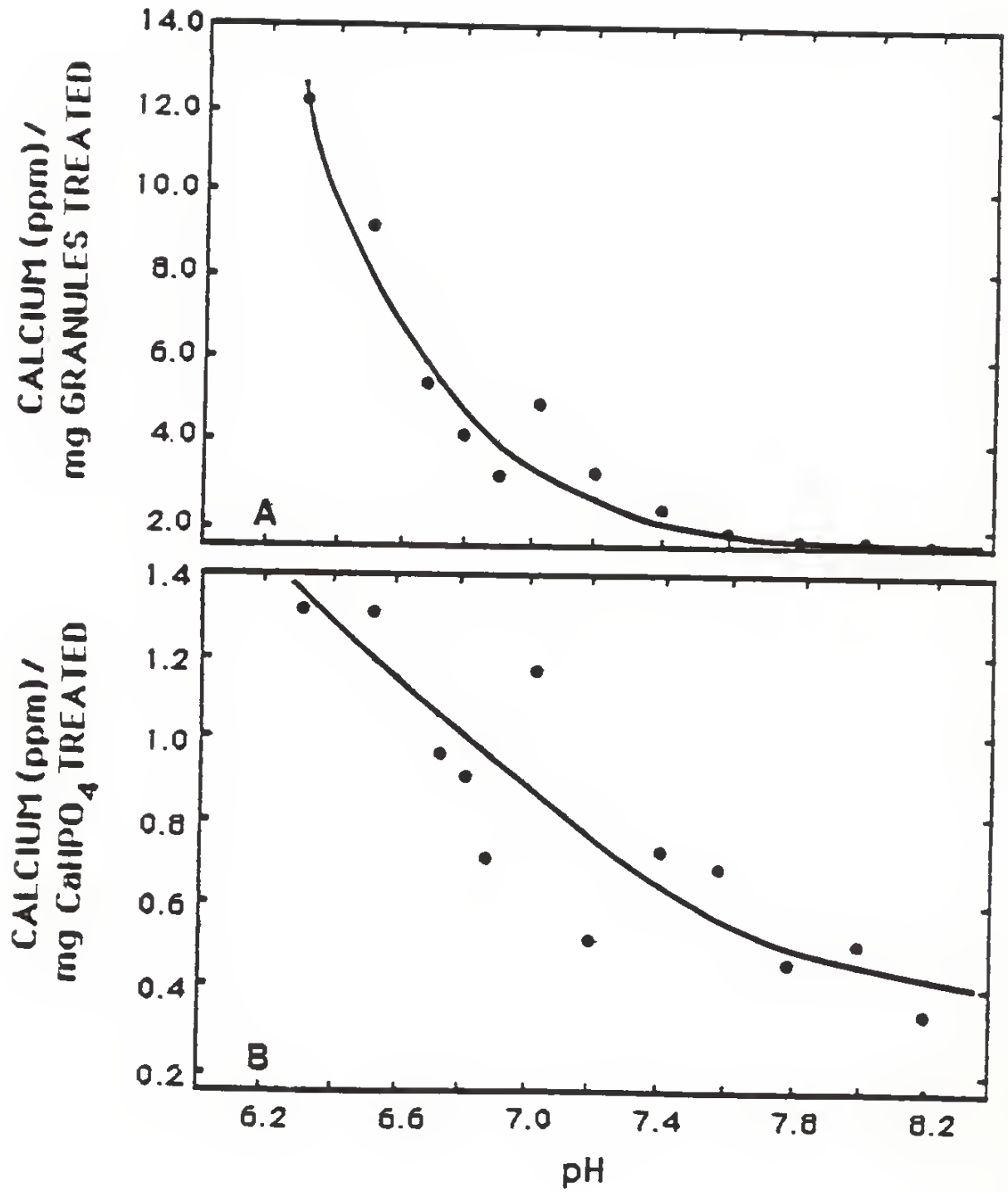
CALCIUM (ppm) / mg GRANULES TREATED
14.0
12.0
10.0
8.0
6.0
4.0
2.0
A
CALCIUM (ppm) / mg $CaHPO_4$ TREATED
1.4
1.2
1.0
0.8
0.6
0.4
0.2
B
6.2
6.6
7.0
7.4
7.8
8.2
pH

Table 2. Utilization of calcium resources, stored in the larval face fly, in the adult fly, puparium, and waste products when larvae were reared at the rate of 1 larva/3 g feces.

Compartment	ug Ca/mg Tissue	Tissue Weight (mg)	Total Ca (ug)	% Utilization
Adult fly	23.71 ± 12.60	5.4 ± 0.52	124.50 ± 67.95	14.73 ± 5.7
Waste Products	23.26 ± 18.09	0.29 ± 0.15	6.77 ± 6.70	0.97 ± 1.1
Puparium	178.61 ± 48.63	3.95 ± 0.60	667.88 ±137.54	84.17 ± 5.0

Values are means ± 2SE (standard error), n = 3.

Table 3. Hemolymph Ca45 activity of larvae pulsed to fresh feces in mid-third instar and then returned to labelled feces until wandering stage.

Pupariation stage	Ca45 (cpm)/ul hemolymph ± 2SE*
Wandering	91.37 ± 10.58
Anterior Retraction	152.03 ± 16.94
Early pupal	191.75 ± 34.52
Mid pupal	121.20 ± 41.78
Mid pupal + 3 h	103.20 ± 34.50
Mid pupal + 6 h	104.93 ± 21.68
Late pupal	89.04 ± 10.91
Apolysis	52.68 ± 5.42

*Values are mean Ca45 activity ± 2SE (standard error), n = 3.

Table 4. Minimum percent free calcium in the hemolymph of face fly larvae and pupae by micropartition filtration (Amicon Co., Danvers, MA).

Pupariation stage	% minimum free calcium ± 2SE*
Wandering	79.00 ± 5.60
Anterior retraction	89.25 ± 3.50
Early pupal	78.80 ± 10.80

The values for free calcium are minimum values since the filtration membranes bind considerable calcium depending on the buffer used. n = 3.

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MINERAL MOBILIZATION FROM THE MALPIGHIAN TUBULES FOR HARDENING OF PUPARIAL CUTICLE IN THE FACE FLY, *Musca autumnalis* De Geer

by

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ABSTRACT

The process of dissolution of mineralized granules stored in the Malpighian tubules of the face fly, Musca autumnalis, and the subsequent transport of inorganic constituents for use in mineralization of the puparium, was examined. Changes in granule morphology induced during in vitro experiments were correlated with in vivo granule changes during developmental events. Measurements of pH of the larval Malpighian tubules, hindgut, hemolymph, and cuticle were taken to determine the role of pH in the physiology of mineralization. The route of calcium from the granules in the Malpighian tubules to the cuticle was also traced.

The pH of the proximal portion of the Malpighian tubules was significantly lower (7.35) than that of the distal portion of the tubules (8.08). In addition, in vitro experiments indicated that a pH decrease of this magnitude resulted in both increased mineral release from the granules and increased morphological damage to the granules. Minerals released from the granules were apparently transported directly from the Malpighian tubules to the cuticle via the hemolymph. The hindgut and rectum were not involved in transport during the larval-pupal transformation stages. A total of 0.6 - 1.0 mg of calcium was transported, in a steady state process, to the cuticle with no significant changes in the calcium concentration or osmolality of the hemolymph. Most of the minerals stored in the larval stages were utilized in the

puparia with minor amounts being utilized in the adult fly, and some excreted as waste. The deposition of minerals in the puparial cuticle was accompanied by an increase of cuticle pH from 7.04 at wandering stage to 8.4 by the early pupal stage.