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A MALPIGHIAN TUBULE LIME GLAND IN AN INSECT INHABITING ALKALINE SALT LAKES

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Summary

The alkali fly, *Ephydra hians* Say, inhabits alkaline salt lakes which can contain concentrations of dissolved carbonate and bicarbonate as high as 500 mmol l^{-1}. Larvae of the alkali fly possess two pairs of Malpighian tubules. The posterior pair has a morphology similar to that of the tubules of most other insects, but the anterior pair is modified into an enlarged gland containing white microsphere concretions. We describe the ultrastructure of all cell types in both pairs of tubules. Using scanning electron microscope (SEM) X-ray microanalysis and chemical CO_2 quantification, we demonstrate that the concretions in the lime glands are composed of nearly pure calcium carbonate. Isolated preparations of lime gland tubules accumulate ^{45}Ca significantly more rapidly than do normal tubules. Although similar to the lime concretions found in the Malpighian tubules of other Diptera, the lime glands of this insect may function to regulate the high concentrations of carbonate and bicarbonate encountered in their aquatic environment. It is proposed that the mechanism of this regulation may be chemical precipitation of carbonate/bicarbonate with calcium in the lumen of these specialized lime gland tubules.

Introduction

Larvae of the alkali fly *Ephydra hians* (Diptera: Ephydridae) are found primarily in hypersaline alkaline lakes throughout western North America (Aldrich, 1912; Wirth, 1971; Herbst, 1986). This habitat preference, and limited ability to survive and develop in non-alkaline water (Herbst *et al.* 1988; D. B. Herbst, unpublished observations), suggests a physiological specialization for living in the unusual chemical environment of alkaline salt lakes. These lakes are usually enriched in carbonate and bicarbonate, often with a pH of 10 or above. *E. hians* larvae are osmoregulators (Herbst *et al.* 1988), capable of maintaining hemolymph osmolality in media with an osmotic concentration 10 times higher

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than that of the blood. Sodium and chloride make up approximately 70% of the total hemolymph osmolality over a wide range of salinities (Herbst *et al.* 1988), and blood pH is approximately neutral (6·5–7·0, D. B. Herbst, unpublished data).

E. hians larvae possess a pair of modified Malpighian tubules containing large quantities of a white granular substance in addition to another pair that do not contain this substance. Herbst *et al.* (1988) noted that the white granules dissolved in dilute acid with the release of gas bubbles, suggesting the possibility that the modified tubules may store carbonate.

The objectives of the present study were to provide a morphological and ultrastructural description of the Malpighian tubules of alkali fly larvae, comparisons with other dipteran tubules, and an analysis of the composition of the white granular substance of the modified tubules. When the results of our study revealed that modified tubules contained nearly pure $CaCO_3$, we conducted a comparison of the calcium-accumulating capabilities of the modified and unmodified tubules.

Materials and methods

Third (final) instar larvae were collected from the shallow rocky margins of Mono Lake, California in autumn 1986. Larvae were maintained in the laboratory in filtered lake water (total dissolved solutes = 90 g l^{-1}, pH 10, and $HCO_3^- + CO_3^{2-} \approx 500$ mmol l^{-1}). Food was provided in the form of an algal mat collected in the lake at the sediment–water interface and cultured in the laboratory. This is the natural food source of larvae and consists mainly of diatoms, filamentous cyanobacteria and detritus.

Morphology and ultrastructure of the tubules

Tubules were dissected in insect saline containing (mmol l^{-1}): NaCl, 150; $NaHCO_3$, 10; glucose, 34; KCl, 7; and $CaCl_2$, 5; pH 6·9. Light microscopic examination of the tubules was carried out using a Wild M5A stereoscopic microscope or an Olympus BH2 compound microscope. All light microscopy was conducted on intact, unfixed, unstained tubules.

For electron microscopy, tubules were dissected in the above artificial hemolymph and fixed for 1 h in 0·1 mol l^{-1} sodium cacodylate buffer (pH 6·9) containing 4% glutaraldehyde and 0·4 mol l^{-1} sucrose. Following rinsing in cacodylate buffer, the tubules were postfixed for 1 h in 1% OsO_4 in cacodylate buffer, dehydrated in an ethanol gradient, rinsed twice in propylene oxide, infiltrated with Polybed 812 (Polysciences) and polymerized at 60 °C. Blocks were sectioned with a diamond knife. Sections stained with uranyl acetate and lead citrate were viewed and photographed in a JEOL C100 electron microscope. Following fixation in glutaraldehyde and osmium, some preparations were treated with a 1:1 mixture of cacodylate buffer and 0·35 mol l^{-1} citric acid to remove the lime gland crystals. This greatly facilitated sectioning. Comparison with untreated tubules revealed no changes in cell ultrastructure as a result of this treatment.

Isolation of granules and SEM X-ray analysis

Larvae were dissected in artificial hemolymph and the Malpighian tubules containing the white granular deposits were removed, rinsed in distilled water, and punctured so that only the contents were released onto an aluminum SEM holding stub. These samples were dried in a desiccator under vacuum for 24 h and then sputter-coated with gold.

Energy-dispersive X-ray microanalysis of these samples was performed using a Hitachi S-500 scanning electron microscope equipped with a Tracor-Northern 2000 X-ray detector, with both beryllium and thin windows. This technique permits the analysis of elemental composition in samples being examined by SEM (Goldstein *et al.* 1981). Reference samples of $CaCl_2$ and $CaCO_3$ were dispersed in water on SEM stubs, dried and subjected to the same X-ray microanalysis. Detection limits were determined using this method by combining $MgCl_2$ in known ratios with $CaCl_2$.

In addition to the above method of compositional analysis, the carbonate content of the unknown Malpighian tubule concretions was determined by acidifying the dried granules in a closed syringe and injecting the evolved gas into an Ametek CO_2 gas analyzer which had been calibrated with gas samples of known CO_2 content. The volume of carbon dioxide released from a known mass of the unknown substance was compared with that released from standard calcium carbonate treated in the same way.

In vitro *assay for calcium accumulation rates in Malpighian tubules*

The rate of accumulation of calcium by the Malpighian tubules of *E. hians* larvae was compared in lime gland and normal tubules by exposing both pairs of tubules excised from the same individual to ^{45}Ca (added as $CaCl_2$, 35 000 counts min^{-1} $nmol^{-1}$ calcium) in a 100 μl droplet of insect saline under mineral oil (Fig. 1). ^{45}Ca accumulation was determined at 23 °C during incubation periods of

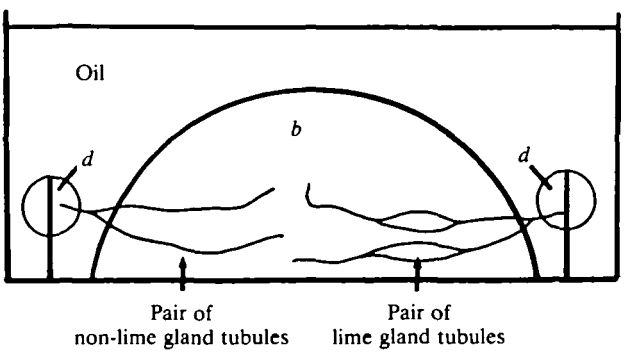

Fig. 1. Diagram of the *in vitro* Malpighian tubule assay system for determining calcium accumulation rates. Excised tubule pairs are suspended in a saline bathing droplet (*b*) isolated under mineral oil. The cut ends of the tubules are pulled out and allowed to secrete droplets (*d*) onto glass posts.

30–60 min. Tubules were removed from the bathing droplet, rinsed in unlabeled artificial hemolymph, and placed in a small volume of 0·1 mol l^{-1} HCl to dissolve the crystals. The total sample was placed in Aquasol-2 (DuPont) scintillation cocktail and counted in a scintillation counter. The amount of medium adhering after removal and rinsing was determined by repeating this rinsing procedure with other tubules in [^{14}C]inulin-labeled artificial hemolymph. This was used as a correction factor for ^{45}Ca contained in medium adhering to the tubules. The calcium accumulation rates per length of tubule were compared statistically using Wilcoxon's signed rank test for paired comparisons of the tubules from within each larva.

Results

Malpighian tubule morphology

White glands, which can be seen through the transparent portions of the cuticle in intact larvae (Fig. 2), are a prominent feature of the larval flies, visible even to the naked eye. Dissection of the larvae reveals two pairs of Malpighian tubules (Fig. 3). Each pair is joined by a common ureter that empties into the gut.

One pair of tubules runs anteriorly into the body and these are modified and divided into three distinct regions (from ureter to tubule tip): proximal, storage and distal regions. (1) The proximal region is a short segment that connects to the ureter. (2) The storage region contains white granular concretions that accumulate throughout larval life and are discharged *via* the gut at pupariation. The granular deposits are microspheres of variable size, up to about 10 μm in diameter (Fig. 4). (3) Distal to the storage region is a long segment that curves back to the posterior

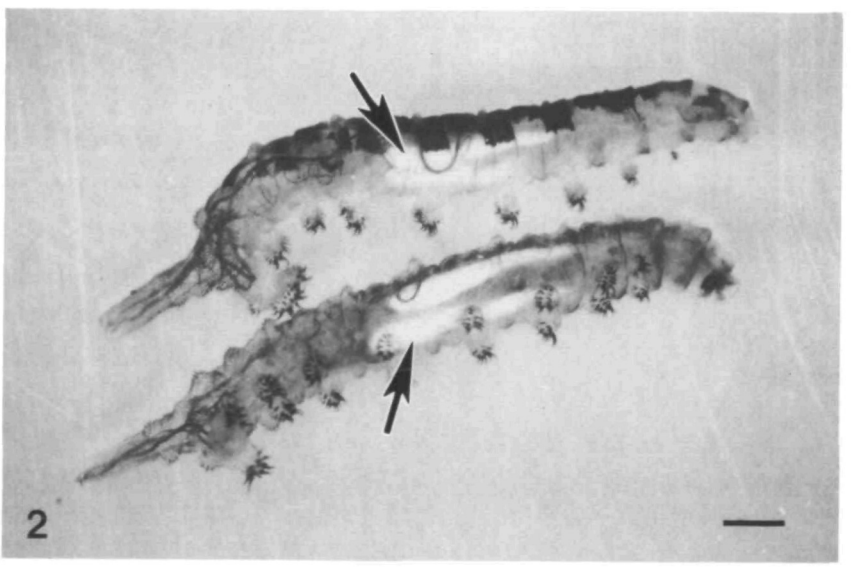

Fig. 2. Third instar larvae of *Ephydra (Hydropyrus) hians*. Arrows indicate position of the modified Malpighian tubules (lime gland). Scale bar, 1 mm.

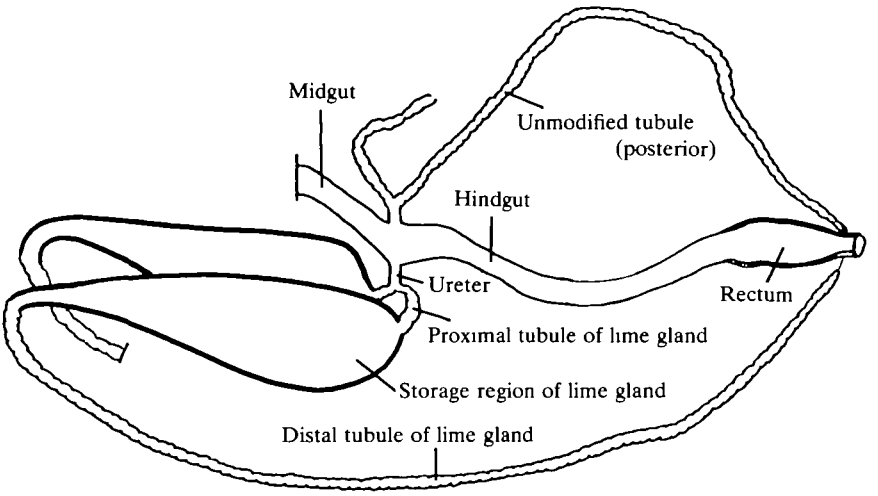

Fig. 3. Schematic diagram of the Malpighian tubules of *Ephydra hians*, showing both the modified pair of lime gland tubules (anterior) and the normal pair of unmodified tubules (posterior).

and also contains some white concretions. The linear dimensions of the tubule segments described here are given in Table 1.

The unmodified pair of tubules run posteriorly and contain no granular deposits. This pair of tubules can be differentiated into proximal and distal segments on the basis of a slight color difference in the natural, unstained condition. The blind distal ends of all tubules are attached by a thin fiber of connective tissue to the outer wall of the rectum. The linear dimensions of the unmodified tubule and its ureter are given in Table 1.

Malpighian tubule ultrastructure

Unmodified tubules

When viewed in the light microscope, the unmodified tubules appear to consist of two regions which can be differentiated on the basis of cell color. The cells in the

Table 1. *Malpighian tubule dimensions*

	Lime gland tubule segments				Unmodified tubule	
	Ureter	Proximal	Storage	Distal	Ureter	Tubule
Length (mm)	0·2	0·5	3·5	6·5	0·2	5·2
S.D.	0·1	0·1	0·5	0·6	0·1	0·9
N	10	12	16	16	9	9

The mean length, standard deviation (S.D.) and sample size (*N*) for third instar larvae, 8–10 mm in body length.

The demarcation between the storage and distal segments of lime gland tubule is the point where the tubules bend posteriorly.

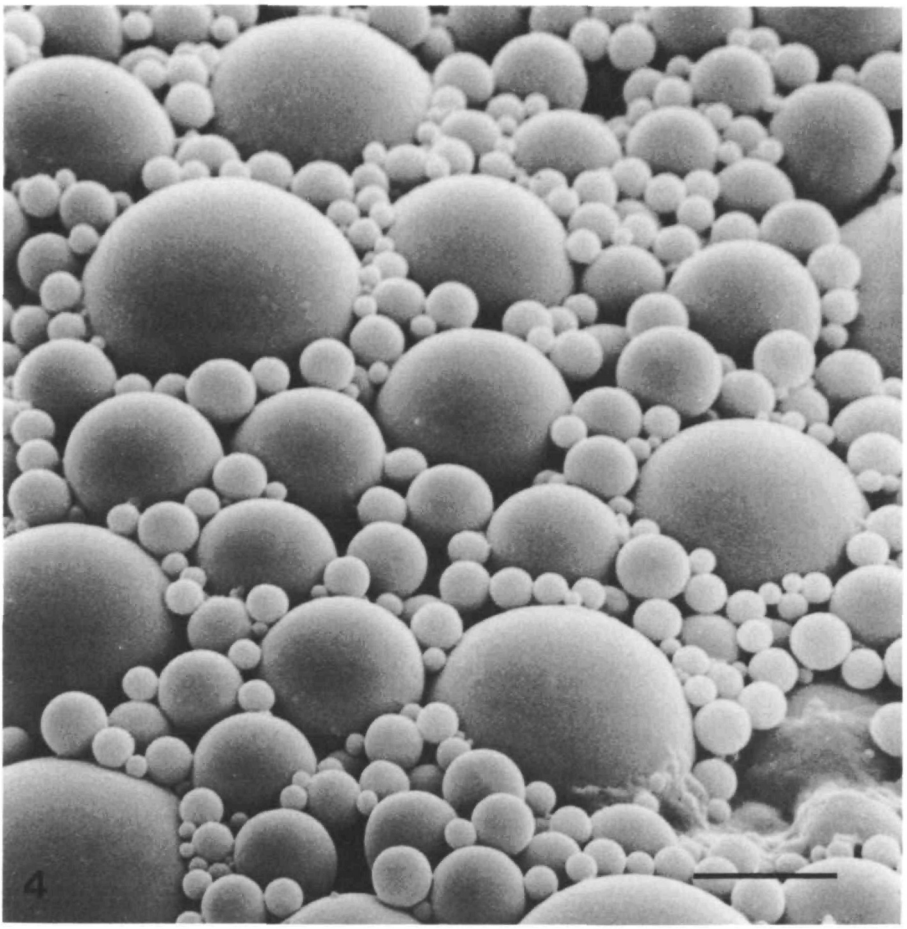

Fig. 4. Scanning electron micrograph of the white microsphere concretions from the lumen of the lime gland Malpighian tubule. Scale bar, 5 μm.

proximal region are clear while those in the distal region appear yellow in the unstained condition. Our ultrastructural examination of cells from these two regions failed to reveal any substantial ultrastructural differences associated with position in the tubule. Throughout the tubule, the epithelium consisted largely of primary cells and of smaller and more infrequent secondary cells (Fig. 5). The primary cells can be up to 20 μm thick, as measured from the basal lamina to the tips of the microvilli, in the regions near the nuclei, but are narrower, about 11 μm in non-nuclear regions (Fig. 5). The microvilli, which are long and closely aligned, frequently contain mitochondria within the microvillar core. The basal infolds are narrow and the cells contain numerous vacuoles. It may be that differences in the content of the vacuoles in the two regions of the unmodified tubules account for the color differences observed in the light microscope.

Interspersed among the primary cells, the tubules also contain secondary cells

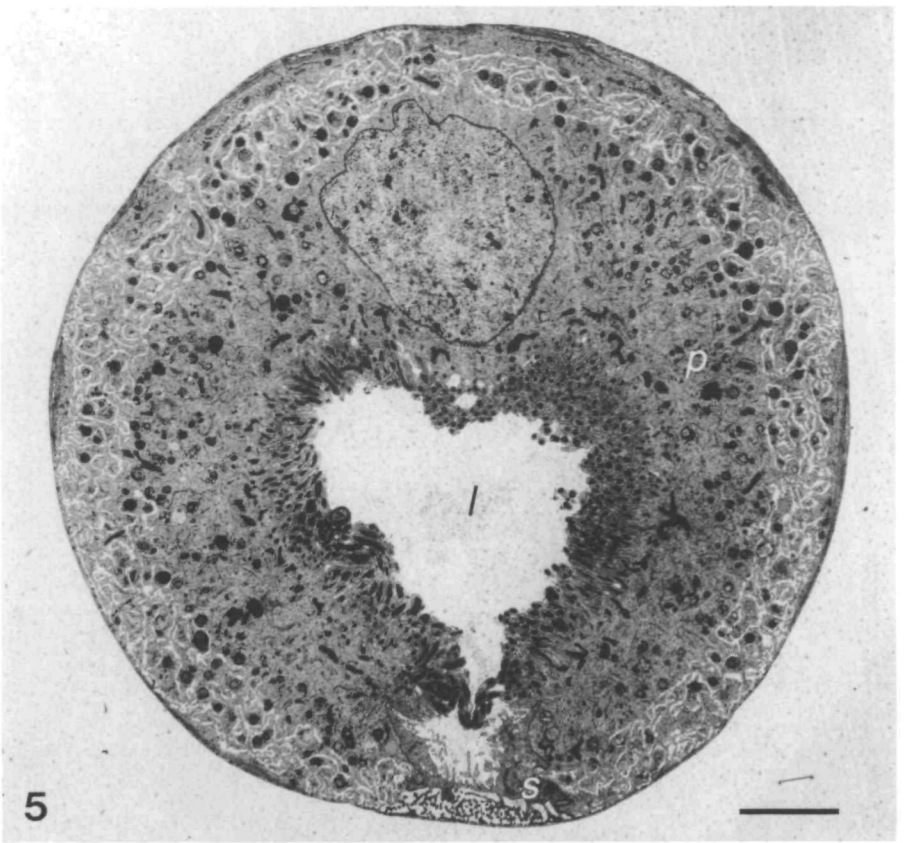

Fig. 5. An electron micrograph of a cross-section of a distal region of a non-lime gland tubule containing both a primary (*p*) and secondary (*s*) cell. *l*, lumen. Scale bar, 5 μm.

(Fig. 6) similar to those described in numerous other dipteran Malpighian tubules (Berridge & Oschman, 1969; Bradley *et al.* 1982; Satmary & Bradley, 1984; Sohal, 1974). In some Diptera, e.g. *Calliphora* and *Aedes*, the secondary cells are termed stellate cells because of the presence of a central cell region containing the nucleus and thin radiating cytoplasmic arms resembling the rays of a star. In *E. hians*, the secondary cells lack these radiating arms and we have therefore chosen the more conservative term of secondary cells for them. The secondary cells are very thin, about 4 μm from basal lamina to microvillar tip. The microvilli are much smaller than those in the primary cells and never contain mitochondria. The secondary cells are found in every region of the tubules of *E. hians*.

Lime gland tubules

Ultrastructural examination of the storage region of the lime gland reveals primary cells that are very different from those in the unmodified tubules (Fig. 7). This tubule segment is highly distended and the cells are thus very narrow in cross-section (Fig. 8). The cells have deep basal infolds and numerous microvilli,

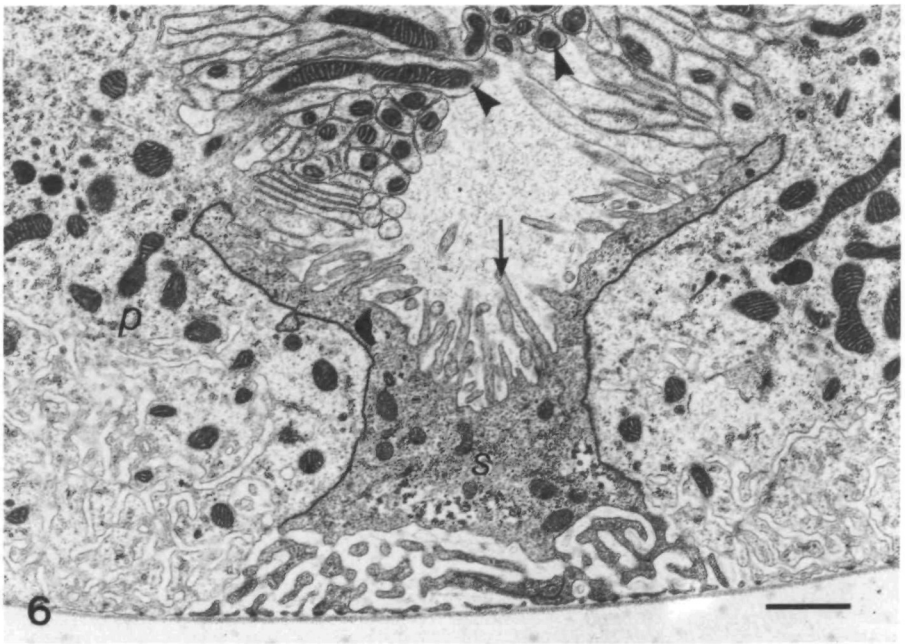

Fig. 6. A secondary cell (*s*) from a non-lime gland tubule. Note that the microvilli (arrowheads) in the primary cell (*p*) contain mitochondria while those of the secondary cell do not (arrow). *l*, lumen. Scale bar, 1 μm.

relatively few with mitochondria in the microvillar core. This region also contains secondary cells (not shown) which are flattened. Distal to the storage region, the tubules are less distended but the cell types are essentially identical with regard to microvillar size, mitochondrial distribution, basal infold length and vacuolar content to those observed in the storage region (Fig. 9). We conclude that the storage region and the distal region are of one cell type and that this region distends as granules accumulate, beginning at the proximal end of the storage segment and proceeding distally.

The proximal region of the lime gland tubules contains primary cells which are distinct in type from those of the distal and storage segments of the same tubule (Fig. 10). The cells are much thicker, and have numerous, closely aligned microvilli which frequently contain mitochondria. The ultrastructural features of these cells are quite similar to those of cells in the proximal and distal regions of the unmodified tubules.

Composition of the Malpighian tubule concretions

The energy-dispersive X-ray microanalysis of the microsphere concretions shown in Fig. 4 revealed a predominance of calcium, with traces of magnesium (Fig. 11). Determinations of the detection limit for magnesium suggest that the crystals contain only 5 % as much magnesium as calcium. The calcium signal from

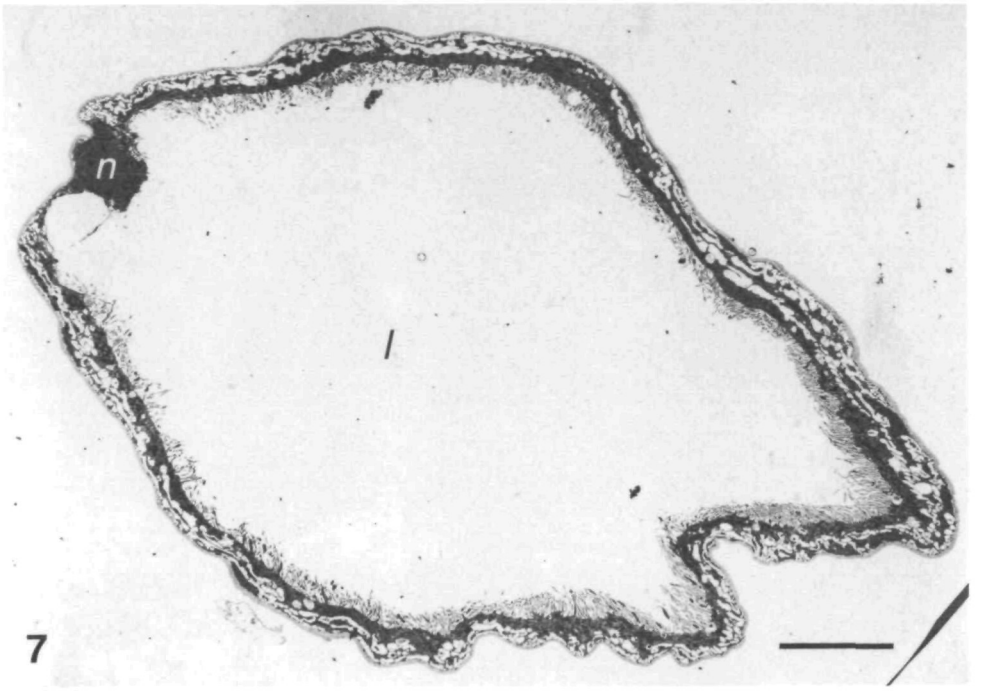

Fig. 7. A cross-section of the storage region of a lime gland tubule. *n*, nucleus; *l*, lumen. Scale bar, 10 μm.

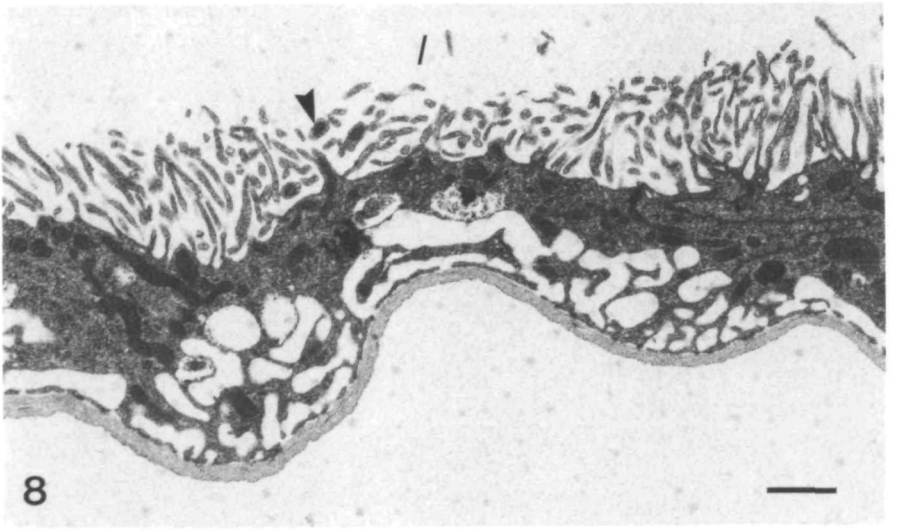

Fig. 8. A higher magnification electron micrograph of the epithelium in the storage region. Some of the microvilli contain mitochondria (arrowhead). *l*, lumen. Scale bar, 1 μm.

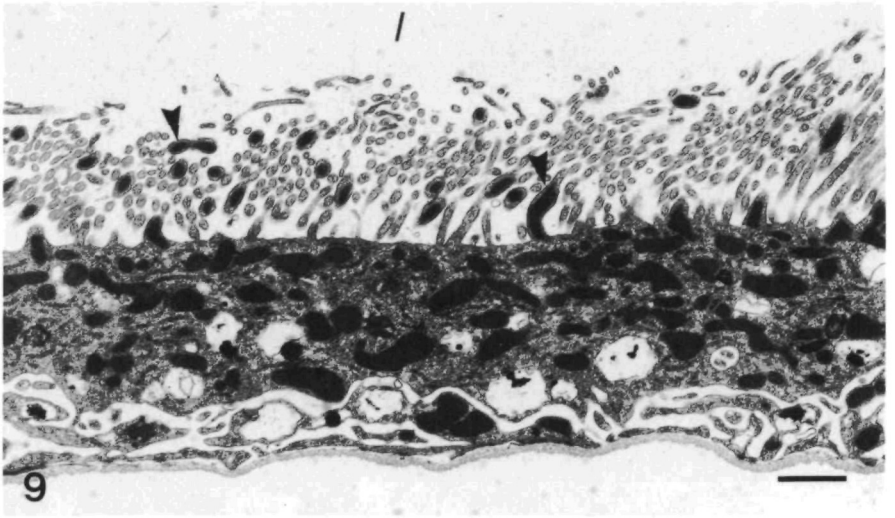

Fig. 9. An electron micrograph of the distal region of a lime gland tubule. Some microvilli in this region also contain mitochondria (arrowheads). *l*, lumen. Scale bar, 1 μm.

the concretions matches that of the $CaCl_2$ reference. However, the strength of the calcium signal at low energy levels obscures signals that would have derived from carbon and oxygen. Therefore, the presence of carbonate was tested for by comparing the amount of carbon dioxide generated after adding excess acid to known weights of dry tubule concretions and standard $CaCO_3$. The amount of carbon dioxide produced by the tubule concretions was compared with that produced by pure calcium carbonate (Fig. 12). The results indicate that the crystals contain a quantity of carbon dioxide equivalent to 84 % of that in pure $CaCO_3$.

Calcium accumulation by isolated Malpighian tubules

The *in vitro* preparations of Malpighian tubules in artificial hemolymph showed that lime gland tubules accumulate ^{45}Ca significantly more rapidly than the normal Malpighian tubules (non-parametric paired-comparison test). There is no overlap in the frequency distributions of calcium accumulation rates between the two tubule types (Fig. 13).

Discussion

The present results show that the anterior Malpighian tubules of the alkali fly *Ephydra hians* have specialized storage segments that are modified into lime glands. These segments contain microspheric crystals that are formed of nearly pure calcium carbonate.

The lime gland of the alkali fly is present throughout larval life. The size of the

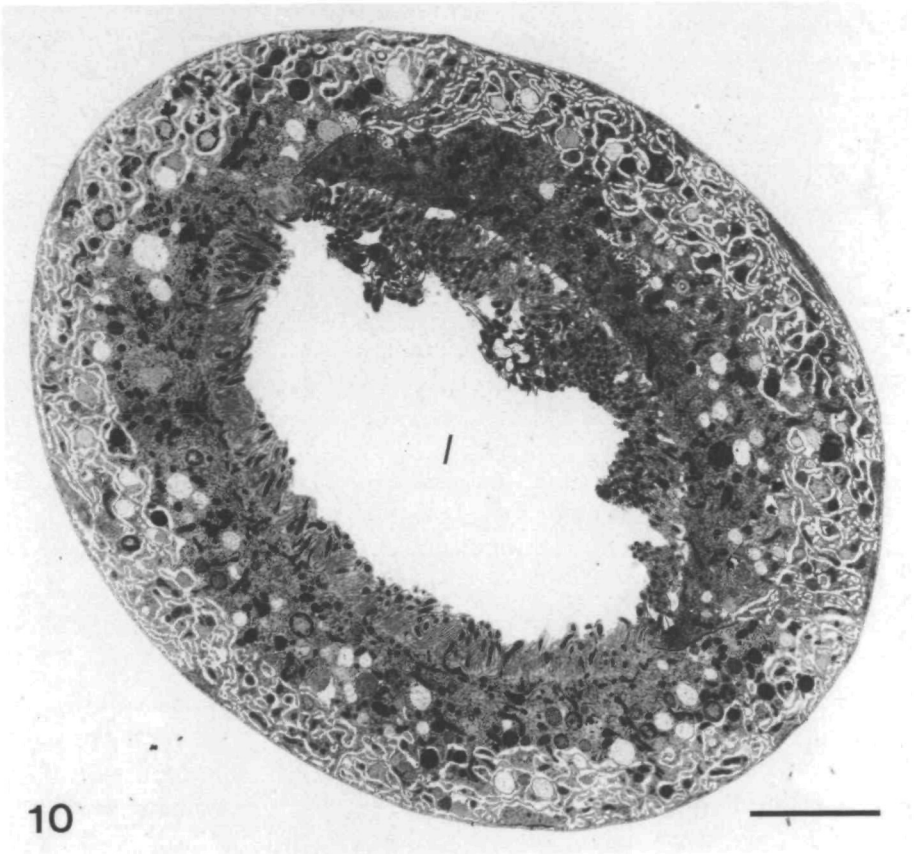

Fig. 10. The proximal region of a lime gland tubule. *l*, lumen. Scale bar, 5 μm.

gland increases as larvae grow, and the concretions accumulate over the three instars. This gland may thus be regarded as a storage–excretion organ. Only at pupariation are the contents of the lime gland discharged into the gut and voided. The timing of elimination of lime from Malpighian tubules in other Diptera has also been found to coincide with molt to the pupa (e.g. Keilin, 1921; Grodowitz & Broce, 1983), when lime dissolves and is deposited onto cuticle, or adult eclosion (e.g. Eastham, 1925; Waterhouse, 1950), when lime granules are excreted along with the meconium.

The anatomy of lime gland Malpighian tubules in the Diptera is variable. In *E. hians*, (as in *Drosophila hydei*, *Ephydra riparia*, *Lucilia cuprina* and *Musca autumnalis*), it is the joined pair of anterior tubules that contain concretions, whereas it is one tubule of each pair that is so modified in *Drosophila melanogaster* (Wessing, 1962). In most of these species it is the distal tubule that is enlarged as the storage region, in contrast to the medial storage region in alkali fly larvae (also found in *Ptychoptera* larvae, Pantel, 1914).

Although the microsphere concretions of *E. hians* are similar in form to those

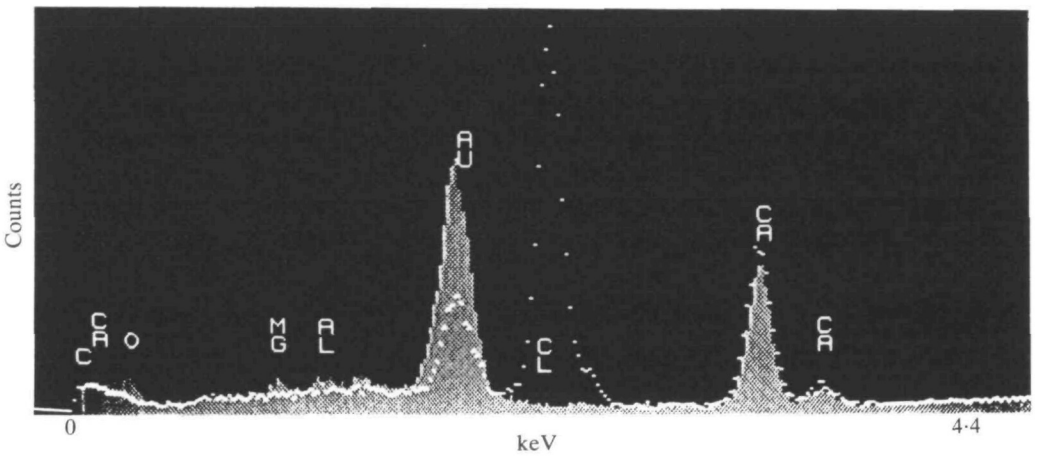

Fig. 11. Energy dispersive X-ray microanalysis of the concretions shown in Fig. 4. Gold and aluminum in the energy spectrum are associated with the sputtered coating and holding stub, respectively. Dashed white lines indicate the spectrum for a $CaCl_2$ reference. Only calcium is clearly present, but magnesium and oxygen also give signals above background level.

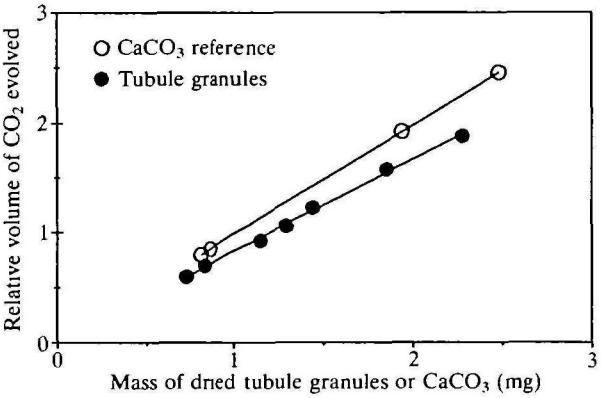

Fig. 12. Comparison of CO_2 gas analysis for the gas evolved upon acidification of dried concretions from the modified Malpighian tubules of *Ephydra hians* and reagent grade $CaCO_3$.

observed in other dipterans such as *Musca autumnalis* (Grodowitz & Broce, 1983), *Lucilia cuprina* (Waterhouse, 1950) and *Drosophila hydei* (Hevert, 1975), the composition differs. In these other dipterans there are substantial amounts of phosphorus, magnesium and other elements, whereas in *E. hians*, the concretions are essentially pure calcium carbonate, with only traces of magnesium present. The spherical appearance of the lime concretions differs from that of calcium carbonate crystals of nonbiological origin, suggesting that they may also contain some organic emulsifier associated with the surface of the precipitate.

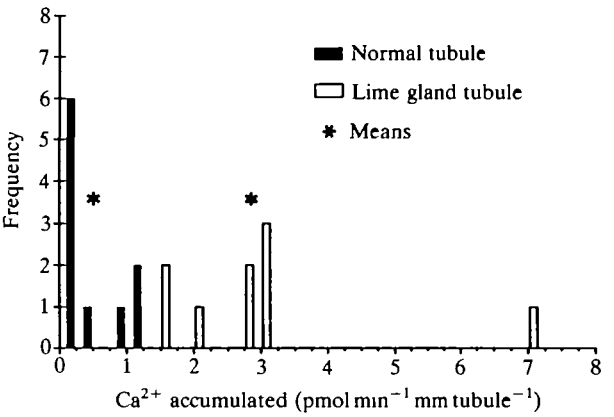

Fig. 13. Frequency distribution of ^{45}Ca accumulation rates in lime gland tubules compared with normal tubules using the *in vitro* tubule assay system. Asterisks are located over the means for the two tubule types. Paired comparisons for the two tubule types taken from the same individual showed higher rates of accumulation in lime gland tubules in all cases. $N = 9$ for lime gland tubule; $N = 10$ for normal tubule.

Lime gland tubules in *E. hians* accumulate ^{45}Ca significantly more rapidly than do the unmodified Malpighian tubules (Fig. 13). Calcium accumulation by the lime gland suggests a possible mechanism for the removal and regulation of environmental CO_3^{2-}/HCO_3^- by chemical precipitation. Since the calcium concentration is low in Mono Lake water ($0{\cdot}1\,mmol\,l^{-1}$) compared to the larval hemolymph ($5–10\,mmol\,l^{-1}$), calcium must be accumulated against a gradient to enter the hemolymph compartment. Although it is not clear that there is further active uptake into the compartment represented by the lumen of the lime gland Malpighian tubule, there is clearly more calcium accumulated across the epithelium of the lime gland than in the normal tubules. Movement of calcium into the lime gland may serve to precipitate carbonate and bicarbonate within the tubule lumen and thus remove these anions from the blood by passive entry into the lime gland down a concentration gradient. The K_{sp} (constant solubility product) of calcium carbonate is about 1×10^{-8}, and that for magnesium carbonate is about 2×10^{-5}, indicating that it is far more favorable for the precipitation of carbonate to use calcium rather than magnesium. Active transport of either the cation Ca^{2+} or the anions CO_3^{2-} and HCO_3^- would serve to set up both an electrical and a chemical inward gradient for the counterion. Our present data are insufficient to determine which ions are actively transported.

The cell types present in the Malpighian tubules of *E. hians* are not different from those of other Diptera (house flies, Sohal, 1974; mosquitoes, Bradley *et al.* 1982; blowflies, Berridge & Oschman, 1969). All regions of the tubules possess primary cells and secondary cells. The primary cells in the tubules of other species have been proposed as the cell type involved in fluid secretion by active ion transport into the tubule lumen, while the stellate cells are thought perhaps to be a

site of urine modification by ion exchange (Berridge & Oschman, 1969; reviewed by Bradley, 1985). Although the normal tubules in *E. hians* possess two regions which differ slightly in color, electron microscopic examination indicates no ultrastructural differences.

The lime glands are also composed of primary and secondary cells. The primary cells in the storage region are flattened, presumably as a result of stretching of the epithelium as lime concretions accumulate. The primary cells of the distal lime gland tubules are less flattened, but have smaller and more widely spaced microvilli containing fewer mitochondria, than the primary cells found in the normal tubules. In general, the secretory cells of insect Malpighian tubules that are engaged in rapid fluid secretion tend to have large microvilli with more numerous intramicrovillar mitochondria (reviewed by Bradley, 1985). The ultrastructure of the primary cells in the tubules of *E. hians* would therefore suggest that the normal tubules are engaged in fairly rapid ion transport and fluid secretion, while ion and fluid transport in the distal tubule of the lime gland may be less vigorous. The distal tubule of the lime gland in *E. hians* contains small amounts of lime concretions. It seems possible, therefore, that calcium carbonate forms in this distal region and is then moved into the storage region. Testing this hypothesis, based on the ultrastructure of the tubules, must await the detailed physiological examination of secretory and resorptive events in the various segments of the tubules. Our results indicate that the distal region of the lime gland tubules is a site of rapid Ca^{2+} flux across the epithelium, and it may be that transport of other ions, and therefore of fluid, is lower in the lime gland tubules than in the normal tubules. This suggestion is supported by the observation that the lime glands are often packed with lime granules, making rapid fluid passage down the tubule impossible. Furthermore, *in vitro* preparations of normal tubules will secrete readily, whereas lime gland tubules secrete fluid very slowly, if at all (D. B. Herbst & T. J. Bradley, unpublished observations).

The formation and storage of calcium-containing deposits in the lumen of Malpighian tubules has been reported from a variety of dipterans (Keilin, 1921; Eastham, 1925; Waterhouse, 1950; Grodowitz & Broce, 1983; Wessing & Eichelberg, 1975). The chemical composition of these granular deposits is variable, but they often contain magnesium, phosphate, carbonate and an organic component, in addition to calcium. Although the role of insect Malpighian tubules as storage–excretion organs for nitrogenous waste in the form of urate crystals is a well-known phenomenon (Maddrell, 1971), the mechanism and functional significance of calcium deposition is poorly known. Hevert (1975) has suggested that the formation of concretions in the lumen of Malpighian tubules in *Drosophila hydei* may be associated with the excretion of excess dietary calcium. Calcium stored in the Malpighian tubules of the face fly *Musca autumnalis* (Grodowitz & Broce, 1983), and in other dipterans (Keilin, 1921), has been implicated as a source for calcium that becomes deposited onto the cuticle during puparium hardening. As with the precipitation of uric acid crystals, calcium concretions are usually confined to specific regions of certain tubules, suggesting a localization of

specialized secretory cells. Although the present research demonstrates specialized morphology, content and activity of lime glands in *E. hians*, further study is needed to determine their functional significance in relation to the environment of this insect.

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