Transport of the cationic fluorochrome rhodamine 123 in an insect's Malpighian tubule: indications of a reabsorptive function of the secondary cell type

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Summary

The pathway of rhodamine 123 was examined after injection into Sarcophaga flies and after in vitro labeling of the Malpighian tubules. After in vitro labeling the primary cells only retained this potential-sensitive dye for a short period while all secondary cells accumulated the dye from the tubule lumen. In vivo the secondary cells also accumulated rhodamine 123 from the lumen, but the primary cells in the distal parts of all four tubules retained the dye for prolonged periods. This was most pronounced in the distal part of the anterior Malpighian tubules, where rhodamine 123 was eventually precipitated on the luminal concretions. Rhodamine 123 initially accumulated in the secondary cell mitochondria and eventually in intensely fluorescing vesicles, probably lysosomes. No evidence for endocytotic processes from the lumen was found using Lucifer Yellow CH, fluorescent dextrans and fluorescent albumin. Prior incubation with the ionophores valinomycin, nigericin, CCCP (all 1 µg/ml), dinitrophenol (1 mM) and NaN3 (10⁻² M) inhibited the selective accumulation of rhodamine 123 to a large extent while monensin (1-5 µg/ml) showed little inhibitory effect. Furthermore, only cationic and no anionic or neutral dyes were accumulated by the secondary cells. In the fleshfly Calliphora and the fruitfly Drosophila, the dye rhodamine 123 also selectively accumulated in the secondary cells, as well in vitro as in vivo.

Key words: Malpighian tubules, dye transport, reabsorption, secondary cells, rhodamine 123.

Introduction

The Malpighian tubules are the so-called 'kidney tubules' of the insects. Extensive research has been devoted to the study of the mechanisms underlying the primary urine secretion by Malpighian tubules (Maddrell, 1978a; Bradley, 1985; Maddrell and Overton, 1990). It has been shown that along with the large amount of fluid passing through or in between the cells, many small molecules also enter the primary urine (Maddrell and Gardiner, 1974; Bradley, 1985). Small essential molecules like amino acids or sugars leaking into the lumen would then be reabsorbed along their passage through the long Malpighian tubules or in parts of the gut such as the rectum. Until now, only an active reabsorption of glucose from the primary urine has been described in the Malpighian tubules of some insects (Knowles, 1975; Rafaeli-Bernstein and Mordue, 1979). Direct uptake of any molecule by a particular cell type in the Malpighian tubules has not been described.

Coloured organic molecules have often been used to characterize the excretory systems of insects. Acidic organic molecules like fluorescein, indigocarmine and amaranth are actively cleared from the haemolymph by the Malpighian tubules, but no accumulation in the cells has been described (Lison, 1937; Maddrell et al., 1974; Bresler et al., 1990). The excretion of basic dyes like neutral red and methylene blue (Lison, 1938; Maddrell, 1977) in some insect Malpighian tubules has been linked to the capability of these tubules to actively excrete toxic alkaloids like nicotine, atropine and morphine, which are also organic bases (Maddrell, 1977).

In this paper we present evidence that in vitro and in vivo the secondary (S) cells of larval and adult Malpighian tubules of some Diptera (Brachycera) are accumulating membrane-permeant cationic dyes but not anionic or neutral dyes from the luminal fluid. Furthermore, in the distal part of the anterior Malpighian tubules rhodamine 123 was precipitated on the luminal concretions.

Besides the Malpighian tubules of the fleshfly Sarcophaga bullata, some comparative investigations were also conducted with the Malpighian tubules of the fruitfly Drosophila melanogaster and a related fleshfly, Calliphora erythrocephala.

Materials and methods

Animals

Sarcophaga bullata (Parker) was reared as described by
Huybrechts and De Loof (1977). In our experiments, male and female flies of day 3 were used, unless mentioned otherwise. *Drosophila melanogaster* was reared as described by Callaerts et al. (1988). The wild type of *Calliphora erythrocephala* was caught in Leuven and reared as described for *Sarcophaga*.

**Supravital staining of the Malpighian tubules**

*In vitro staining of the tubules*

The Malpighian tubules were carefully dissected in Ringer's solution (adapted from Chan and Gehring (1971), in mM: 120 NaCl, 10 KCl, 15 MgSO4, 5 CaCl2, 10 Tricine, 20 glucose, 50 sucrose, pH 6.8). In this solution the tubules could actively secrete fluid for at least 24 hours without oxygen supply, as was measured with the in vitro bio-assay according to Ramsay (1954). Some control experiments were conducted in a glucose-free bicarbonate buffer (*Sarcophaga* Ringer's (in mM): 121.5 NaCl, 10 KCl, 1 NaH2PO4, 10 NaHCO3, 0.7 MgCl2, 2.2 CaCl2, pH 6.8, aerated with 95% O2/5% CO2), according to Verachtert and De Loof (1988).

After staining and rinsing in Ringer's solution, the tubules were transferred to a hollow glass slide in a few drops of Ringer's fluid, and covered with a coverglass. In this covered slide the tubule cells remained viable for many hours (cell viability tested by Trypan blue exclusion; Ashburner, 1989). For immediate photography of these whole-mounts, a flat glass slide with two glass bridges was used.

The fluorescently stained tubules were observed under a Leitz Laborlux S fluorescence microscope, using the rhodamine and fluorescein excitation filters. Rhodamine 123, Lucifer Yellow CH, sulforhodamine B, FITC-labeled dextran of 4.4 and 35 kDa and a TRITC-labeled dextran of 10 kDa were purchased from Sigma. Bovine serum albumin (Serva) was conjugated with FITC (Sigma) as described by Haaijman (1983). Eosins Y and B and rhodamine 6G were purchased from Merck; Pyronin Y (G) was purchased from Serva; merocyanin 540 from Fluka; and fluorescein (sodium) from UCB. Fluorescence was recorded using Kodak T MAX 400 ASA film.

*In vitro injection of fluorochromes into the lumen*

The lumen of the proximal half of a freshly dissected tubule was perfused with a short pulse of dye solution by means of small glass capillaries with an outer tip diameter of 25-40 μm. The dye containing Ringer's solution was injected into the lumen until the coloured fluid was seen to leave the other open tubule end. After 1 to 5 minutes of incubation the dye was rinsed with Ringer's solution and the tubule was mounted in fresh Ringer's fluid in a hollow slide, and covered with a coverglass.

*In vitro transport of fluorochromes in the lumen*

For these experiments, whole Malpighian tubules were set up between a fluorochrome solution and a dye-free Ringer's solution in two separate bathing droplets under paraffin oil (see Maddrell and Overton, 1990).

*Intravital staining of the Malpighian tubules*

In these experiments 2 μl of rhodamine 123 (100 μg/ml) or other dyes in Ringer's fluid (0.1-1 mg/ml) were injected into the thorax of adult *Sarcophaga bullata* by means of small glass capillaries with a tip diameter of about 100 μm. In each experiment a control group, injected with Ringer's solution alone, was kept under identical conditions. After the proper incubation time, the tubules of experimental and control flies were removed in Ringer's fluid, and were immediately observed under the fluorescence microscope. Larvae were also injected with 100 μg/ml.

**In vitro application of drugs and antibiotics**

The drugs and antibiotics were tested at different concentrations for 5-120 minutes on Malpighian tubules in vitro. The following provided the best minimum incubation time and lowest concentration for a clearly detectable effect: valinomycin (Janssen Chimica, Beerse, Belgium), 30 min in 1 μg/ml; nigericin (Sigma), 30 min in 1 μg/ml; monensin (Serva), up to 120 min in 5 μg/ml; CCCP (Serva), 30 min in 1 μM; 2,4-DNP (Serva), 30 min in 1 mM; sodium azide (Merck), 30 min in 10⁻² M. Whenever appropriate, the same concentration of the primary solvent acetone or ethanol was included in the Ringer's solution in the control conditions (max. 0.5%). Only tubules with viable cells were used in the experiments.

**Results**

**General morphology and terminology**

The fleshfly *Sarcophaga bullata* (like most other Diptera) contains 4 Malpighian tubules (Fig. 1), 2 oriented anteriorly (anterior Malpighian tubules or AMT) and 2 oriented posteriorly (posterior Malpighian tubules or PMT) in the abdomen. Each AMT contains a morphologically distinct distal part (dAMT), while the

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**Fig. 1. General morphology and orientation of Malpighian tubules in *Sarcophaga bullata* (after Wessing and Eichelberg, 1969). Notice the swollen distal part (d) of the anterior Malpighian tubule (A) and the morphologically homogeneous posterior Malpighian tubule (P), which resembles the proximal part (p) of the anterior tubule (M, midgut, H, hindgut).**
Dye transport in insect Malpighian tubules

PMT is morphologically homogeneous and similar to the proximal part of the AMT (pAMT) (see Fig. 1). Each tubule consists of two cell types, the primary (P) and the secondary (S) cell type. Although in dipteran Malpighian tubules most of the S cells have a stellate cell shape, we prefer naming them secondary instead of stellate cells, since similar cell types in other insect groups are not stellate (Bradley, 1985). In comparison to the P cells the smaller S cells in Diptera have shorter microvilli, which contain little or no mitochondria, and they have a smaller nucleus and a transparent cytoplasm containing little or no granular deposits.

Alkassis and Schoeller-Raccad (1984) named the cells in the distal part of the AMT of larval Calliphora erythrocephala differently. We suggest that these cells are also named S and P cells, since as well as some differences the cells in distal and proximal tubule parts have some apparent characteristics in common: a small nucleus and a transparent cytoplasm in all S cells compared to a granular cytoplasm containing a large nucleus in all P cells. Furthermore, a very similar F-actin cytoskeleton was found in the S and P cells in both parts of the AMT (Meulemans and De Loof, 1990a,b). We will however always indicate whether the cells are found in the distal or proximal part of the AMT.

Supravital and intravital labeling of the Malpighian tubules with rhodamine 123 and other fluorochromes

Rhodamine 123 (Rh 123) is a very fluorescent rhodamine derivative that is known to be non-toxic to vertebrate cells after a short application of 10 \( \mu \text{g/ml} \) (Johnson et al., 1980, 1981; Chen, 1989). This fluorochrome is known to be attracted by the high potential gradient over the mitochondrial membranes in living cells. Experiments with the ion-selective ionophore nigericin (\( \text{K}^+/\text{H}^+ \) exchanger) showed that it is the potential difference across the mitochondrial membranes rather than the pH gradient that attracts the dye molecules (Johnson et al., 1981).

Supravital labeling of the Malpighian tubules

In vitro labeling

Posterior tubules. In a posterior Malpighian tubule stained in vitro in 5 \( \mu \text{g/ml} \) Rh 123 for 10 minutes and washed with the Ringer’s solution for 5 minutes, the mitochondria were well labeled. All P cells displayed an intense labeling while the S cells appeared largely unstained (Fig. 2A). Because of the granular cytoplasm and the extensive labeling of the P cells, separate mitochondria could not be detected. But the high amount of mitochondria in the P cell microvilli was clearly recognized as an intense apical zone upon these cells. This was most obvious when looking at the P cell brush border through the transparent S cells. Also, due to the intensive staining of the underlying P cells the mitochondria in the S cells, which were poorly labeled, were hard to detect.

If such a living Rh 123-stained tubule was left in the Ringer’s solution for about two hours, an entirely different staining pattern was found. Only the S cells were labeled very intensely while the previously well labeled P cells had lost most of the fluorochrome (Fig. 2B). Even the P cells in the most distal part of the PMT lost their fluorescence after two hours in vitro. Separate mitochondria were clearly recognized as tubular vesicles throughout the entire S cell. This pattern resembled that described for vertebrate cultured cells (Johnson et al., 1980). At first this selective staining of the S cells was thought to be an artefact caused by the prolonged incubation of the tubule in the covered hollow slide. However, when the same tubule was again stained in a fluorescent-containing Ringer’s solution the P cells were labeled as intensely as after the first staining. So their mitochondria did not lose the ability to accumulate Rh 123. The dye must have leaked from the P cells to the lumen.

When such a tubule with brightly labeled S cells was left in the Ringer’s solution for another hour or longer, large round vesicles were formed (Fig. 2D). Concomitantly the typical mitochondrial pattern disappeared. Possibly these are enlarged, poisoned mitochondria or lysosomal vesicles that finally accumulated the dye molecules.

Anterior tubules. Immediately after in vitro labeling all P cells in the AMT were brightly labeled, even those in the distal part of the AMT. As in the PMT, the S cells only displayed minor fluorescence. When a tubule was left in the Ringer’s solution for two hours, even the S cells in the distal part of the AMT selectively accumulated the dye (Fig. 2C). After prolonged incubation in Ringer’s solution, the S cells also displayed bright round vesicles in both distal and proximal parts of the AMT. The results of the in vitro staining of both AMT and PMT are summarised in Table 1.

In vitro injection into the Malpighian tubule lumen

Posterior tubules. To test whether the S cells had indeed accumulated the dye from the luminal cavity, proximal parts of posterior Malpighian tubules were luminally injected with a 10 \( \mu \text{g/ml} \) solution of Rh 123 in Ringer’s fluid. After incubating for 1 or 2 minutes, it was obvious that only the S cells had accumulated the fluorochrome; the P cells were not labeled (Fig. 2E). It was even unnecessary to rinse the lumen, since little dye remained in the luminal cavity. If the same tubule was then labeled at its haemolymph side, the P cells were brightly stained. They did not lose their ability to take up the Rh 123 but their apical membrane seemed to be quite impermeable to this cationic fluorochrome. If Rh 123 (10 \( \mu \text{g/ml} \)) was injected into the lumen of the most distal part of the PMT, no selective accumulation in the S cells was noticed. The P cells accumulated most of the dye while the S cells only displayed minor labeling. So only the more proximal S cells accumulated the dye from the lumen in these posterior tubules. The apical membranes of the P cells in the distal part of the PMT seem to be permeable to this cationic dye, while the proximally located P cells were largely impermeable.

Anterior tubules. Injection of the dye solution into the lumen of the proximal part of the AMT resulted in the
Fig. 2. Malpighian tubules were stained with Rh 123 in vitro. (A) Immediately after staining for 10 min in 5 μg/ml; the secondary cells (S cells) remain largely unstained; (B and C) proximal (B) and distal (C) part of AMT, 120 min in Ringer’s fluid after staining; all S cells accumulated the dye while all P cells lost it; (D) S cell, 180 min in Ringer’s solution after staining; the mitochondrial pattern has disappeared and round vesicles are found; (E) Rh 123 (10 μg/ml) was injected into the lumen of the proximal part of a tubule; only the S cells accumulated the dye from the lumen; (F-I) the following fluorochromes were injected into the proximal part of a tubule: 10 μg/ml rhodamine B (F), 20 μg/ml merocyanin 540 (G), 20 μg/ml fluorescein (H) and 20 μg/ml pyronine Y (I). Only the latter (cationic) dye is accumulated by the S cells. The anionic dyes (in G and H) are found in high concentrations between the P cell microvilli (arrowheads). Bars, 10 μm, A, B, D, F-I; 20 μm, C and E.
same pattern: the S cells accumulated the dye from the lumen while the P cells remained unlabeled. Injection into the distal part of the AMT was difficult due to the high amount of granular material in the lumen, but in the few tubules that were successfully injected only the S cells accumulated the dye.

In vitro transport through the Malpighian tubule lumen

To determine if there really was any transport of dye through the tubule lumen, we set up living posterior tubules as a connection between the fluorochrome solution (10 μg/ml Rh 123) and the Ringer's solution (Maddrell and Overton, 1990). It was obvious from these experiments that the Rh 123 was indeed transported through the cells of the distal part (lying in the fluorochrome solution) to the lumen and that the S cells (but not the P cells), which were more proximally located, had accumulated the dye from the lumen. The dye was also found to have accumulated in the S cell mitochondria.

Intravital labeling of the Malpighian tubules

Posterior tubules

About 10 minutes after injection of Rh 123 (100 μg/ml) in intact flies all P cells were heavily stained, while the S cell mitochondria were poorly labeled (Fig. 3A). After 60 minutes or longer post injection the more proximally located S cells were labeled intensely while the P cells had lost their fluorescence in this part (Fig. 3B). Only the P cells in the more distal part of the PMT (Fig. 3C) kept the dye for prolonged periods. The S cells in the distal regions accumulated much less fluorescence compared to those parts in which the P cells had lost the dye. Only about 40 hours after injection into the adult, the dye had left the primary cells of all tubules. In contrast to the AMT, the terms proximal and distal parts of the PMT do not refer to certain clearly recognizable zones. Even in electron microscopical investigations, the PMT of the related fly Calliphora could not be subdivided into different regions (Berridge and Oschman, 1969; Alkassis and Schoeller-Raccaud, 1984). The 'distal part' comprises from about one third to one fifth of the tubule length. There is a gradual transition of stained versus non-stained P cells after prolonged periods post injection the length of the distal part of the PMT that displays brightly labeled P cells decreases and eventually all P cells have lost the fluorochrome. Concomitantly, the S cells in such parts display an increase in fluorescence intensity.

Granular concretions were also found in the lumen of the PMT, but unlike those in the distal part of the AMT (see below) they did not display any detectable fluorescence.

After about 40 hours post injection, the S cells displayed diffuse labeling of the whole cell instead of the mitochondrial pattern. As occurs after prolonged in vitro incubation of a stained tubule, most S cells contained some very fluorescent vesicles in their cytoplasm (Fig. 3D). These vesicles had a round shape and they exhibited an intense fluorescence after both fluorescein and rhodamine excitation. In the intact fly these vesicles were smaller and they displayed a brighter fluorescence than the in vitro formed vesicles; they might be lysosomes that finally removed the dye from the cell.

An identical selective accumulation in the S cells was found after injection of Rh 123 in older flies that were fed liver (besides water and sugar).

After injection of 2 μl of 100 μg/ml Rh 123 into third-instar feeding larvae, different observations were made. The S cells also accumulated the dye from the lumen while the P cells lost the dye after about two hours; but in larval tubules the P cells in the distal part of the PMT also lost the dye rather quickly, while the S cells in this part were also well labeled.

Anterior tubules

Also in the AMT, about 10 minutes after injection of Rh 123 (100 μg/ml) into adults all P cells were heavily labeled, while the S cell mitochondria were poorly labeled. After 60 minutes or longer post injection the more proximally located S cells were labeled intensely while the P cells had lost their fluorescence in this part (Fig. 3B).

Only the P cells in the distal part of the AMT kept the dye for prolonged periods. This part showed the highest fluorescence intensity of all the tubules. The S cells in the distal regions accumulated much less fluorescence than those of the proximal parts. But after prolonged

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### Table 1. Relative fluorescence intensities after in vitro labeling of adult Malpighian tubules

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<th>Time after labeling</th>
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The tubules were stained for 10 min in 5 μg/ml rhodamine 123 and were then incubated for the indicated period in Ringer's fluid (AMT, anterior Malpighian tubule; Dist., distal part; P cell, primary cell; PMT, posterior Malpighian tubule; Prox., proximal part; S cell, secondary cell).

***The S cell is diffusely labeled, but some very intensely fluorescing vesicles are spread throughout the cell. These vesicles contain a higher concentration of label than the previously labeled mitochondria.**
Fig. 3. Injection of Rh 123 into intact flies: (A) 30 min after injection; (B) 24 hours after injection; proximal part of the PMT showing selective accumulation in the S cell mitochondria; (C) same fly 24 hours after injection; more distal part of the PMT showing P cells that retain dye longer while the S cells (S) appear largely unlabeled; (D) 30 hours after injection, showing intensely fluorescing round vesicles in the S cells; (E) distal part of the AMT 30 hours after injection; the lumen contains brightly labeled concretions while the cells appear unstained; (F) granules taken from the lumen of the distal AMT 30 hours after injection, showing a rim of Rh 123 around most granules; (G) 90 hours after injection; Rh 123 is found in the centre of most granules. Bars, 5 μm, F and G; 10 μm, B-D; 20 μm, A and E.
periods post injection, even the mitochondria in some S cells in the distal AMT were labeled more intensely than neighbouring P cells. At only about 40 hours after injection into the adult, the dye had left the primary cells of all tubules.

At about the moment the S cells started losing their mitochondrial pattern, the so-called concretions (Brown, 1982; Sohal et al., 1976) in the lumen of the distal part of the AMT were fluorescently labeled (Fig. 3E). When comparing the granular deposits in the distal part of the AMT lumen with those of control flies, it was obvious that the concretions in the dye-injected flies contained the dye rhodamine 123 in a rim around their periphery (Fig. 3F). After about 90 hours post injection, the concretions were still labeled, but now the fluorescence was found in the central part of the lumen of the distal part of the AMT. The latter granules were clearly larger than those in earlier stages. These granules have also been seen to descend along the lumen of the posterior adult tubules, most S cells contained some very fluorescently labeled vesicles. These vesicles are smaller but intenser as those found 180 min after in vitro labeling.

| Table 2. Relative labeling intensities of the different cell types after Rh 123 injection |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|
|---------------------------------------------|-------|-------|-------|-------|-------|-------|
| 10 min                                      |       |       |       |       |       |       |
| PMT                                         | S cell | +     | +     | +     | +     | +     |
| P cell                                      |       |       |       |       |       |       |
| AMT                                         | S cell | +     | +     | +     | +     | +     |
| P cell                                      |       |       |       |       |       |       |
| 60 min                                      |       |       |       |       |       |       |
| PMT                                         | S cell | +     | +     | +     | +     | +     |
| P cell                                      |       |       |       |       |       |       |
| AMT                                         | S cell | +     | +     | +     | +     | +     |
| P cell                                      |       |       |       |       |       |       |
| 40 h                                        |       |       |       |       |       |       |
| PMT                                         | S cell | +     | +     | +     | +     | +     |
| P cell                                      |       |       |       |       |       |       |

2 µl of Rh 123 at 100 µg/ml was injected into adults. See Table 1 legend for abbreviations.

Selective accumulation of other dyes?
In these experiments only the proximal part of adult posterior Malpighian tubules was used. In experiments in which 10 mg/ml Lucifer Yellow CH in Ringer's solution was injected into the lumen of adult tubules no evidence was found of endocytotic processes occurring in the S cell type. Lucifer Yellow CH is a highly fluorescent naphthalimide dye that normally does not penetrate membranes of living cells (Stewart, 1978) and can be employed as a marker for endocytosis (Swanson, 1989). After injection of FITC- or TRITC-labeled dextrans with relative molecular masses of 4.4, 10 and 35X10³ (10 mg/ml) or FITC-labeled bovine serum albumin (10 mg/ml) in the lumen of the proximal part of a Malpighian tubule no uptake of fluorescence was noticed after 60 minutes of incubation. No endocytosis was detected after incubation in the Ringer's solution or in the complex Schneider's medium (Serva).

Injection of other fluorochromes indicated that only positively charged molecules were accumulating in the proximal S cells. The cationic dyes rhodamine 6G and pyronin Y (Fig. 2I) were also accumulating in the S cells after injection into the tubule lumen (20 µg/ml) and after injection into adults (1 mg/ml). The acid (anionic) dyes eosin Y and B, sodium fluorescein (Fig. 2H), sulphorhodamine B and merocyanin 540 (Fig. 2G) were not accumulating in the S cells after injection into the tubule lumen (10-20 µg/ml) or into adults (1 mg/ml). Also, the neutral dye rhodamine B did not selectively accumulate in the S cells after injection into the tubule lumen (10 µg/ml) or into adults (100 µg/ml; Fig. 2F). This neutral and the acid dyes entered the P cells after

<p>| Table 3. Relative labeling intensities after injection of 2 µl rhodamine 123 (100 µg/ml) into third-instar feeding larvae |</p>
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No long-term experiments were conducted.
injection into the lumen, while the S cells were poorly labeled.

No difference in response to the various dyes was noticed between the sexes.

**Comparison with other Diptera (Brachycera)**
The Malpighian tubules of adult *Drosophila melanogaster* and adult *Calliphora erythrocephala* also showed an accumulation of Rh 123 in their S cells, after in vitro and in vivo labeling. Remarkably, the S cells in the distal part of the AMT also, which are often described as a different cell type, accumulated Rh 123 from the Malpighian tubule lumen.

**In vitro administration of drugs and antibiotics**
In an effort to understand the process of selective accumulation of Rh 123 and other cationic dyes by the secondary cells, some ion-selective ionophores (Pressman, 1976) and drugs that interfere with the metabolism were applied to posterior Malpighian tubules before or after staining with Rh 123.

**Drug administration after in vitro labeling**
When tubules previously stained at their haemolymph side with Rh 123 in vitro were treated with the protonophore 2,4-dinitrophenol (2,4-DNP, 1 mM) or with the inhibitor of electron transport sodium azide (*10^-2 M*), for 30 minutes, the S cells also accumulated the dye after about two hours of incubation in fresh Ringer's solution, but less than in the control. The staining was diffuse, no mitochondria were labeled. The S cell mitochondria lost the dye more rapidly in these tubules, while the S cells still accumulated some dye diffusely throughout their cytoplasm. Administration of the protonophore carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) to tubules previously stained with Rh 123 showed that after 60 minutes in 10 *µM* CCCP, much dye was accumulated by the S cells, although no mitochondria were stained. The fluorescence was spread diffusely throughout the cell, and was also concentrated in some round vesicles in the S cell cytoplasm. Although the mitochondrial membrane potential, and thus after a while also the cell membrane potential, is inhibited in such cells, the dye still accumulated in the S cells to a large extent (although not in the mitochondria).

**Drug administration prior to luminal dye-injection**
Treatment of tubules with several ionophores prior to injection of Rh 123 in the tubule lumen caused the dye to leak from the S cell mitochondria. In the case of CCCP, much of the fluorescence was retained in large round vesicles in the S cells. All the latter treatments are known to abolish the mitochondrial membrane potential (Johnson et al., 1981; Harold, 1986). Together with the stimulation of dye uptake by nigericin, it may be concluded that the S cell mitochondria were the organelles accumulating Rh 123.

**Influence of other parameters**
Injection of Rh 123 into tubules bathed for 60 minutes in Ringer's solution with NaCl, or KCl replaced by equimolar quantities of choline chloride (Serva), had no effect on the accumulation of Rh 123.

Incubating adult tubules in *Sarcophaga* Ringer's fluid (Verachtert and De Loof, 1988) for two hours after in vitro labeling or dissection of tubules in this Ringer's solution from dye-injected flies did not result in any different results. So whenever a possible change in internal pH was caused by the bicarbonate-free Ringer's that we used (Thomas, 1989), this did not result in an inhibition of dye uptake by the S cells. The S cell mitochondria were also brightly labeled in this Ringer's fluid while the P cells were mostly unlabeled.
Fig. 4. Injection of Rh 123 into the Malpighian tubule lumen after addition of drug or antibiotic: little or no dye is accumulated by the S cells (S) after addition of nigericin (A, 1 μg/ml for 20 min), valinomycin (C, 1 μg/ml for 60 min), CCCP (D, 10 μM for 30 min) and 2,4-DNP (E, 1 mM for 60 min), while the S cell mitochondria remain well labeled after 120 min in 1 μg/ml monensin (B). Incubation in nigericin (1 μg/ml for 15 min) after injection of Rh 123 (1 μg/ml) into the tubule lumen shows brightly labeled S cells (F), while another tubule of the same fly, incubated in Ringer’s fluid, shows S cells that accumulated much less dye (G). Bars, 10 μm A-E; 20 μm, F and G.
Incubating a tubule for two hours in Ringer's solution at 4°C prior to injection of cold Rh 123 into the lumen did not decrease the accumulation of Rh 123 into the S cell mitochondria. If a tubule stained in Rh 123 at its haemolymph side was left for three hours in Ringer's solution at 4°C, the P cells were still brightly labeled while the S cells had not accumulated any dye. So the low temperature inhibited the release of the dye molecules from the P cell mitochondria. The S cells can still accumulate the dye from the lumen under this condition, but since little dye was leaking from the P cells they appear largely unstained.

Injection of a pulse of the non-ionic detergent Triton X-100 (0.1% in Ringer's solution) into the tubule lumens prior to injection of Rh 123 (10 μg/ml) decreased the dye accumulation in the S cell mitochondria, but the proximal P cells also were diffusely labeled. So permeabilisation of the S and P cell apical membranes decreases the mitochondrial staining, but the P cells become permeable to the dye molecules.

Incubating a tubule for up to two hours in Ringer's solution containing the cardiac glycoside ouabain (10^-4 M, Serva) prior to injection of Rh 123 into the lumen had no detectable effect on the accumulation of the dye in the S cells.

Discussion

Our experiments show that secondary cells can accumulate some small organic molecules with positive charge (at physiological pH) from the luminal fluid. The primary cell apical membrane in the more proximal parts of the tubules seems to be largely impermeable to these cationic dyes. As seen in the experiments with CCCP, 2,4-DNP and NaN3, the large fluid flow through the P cells towards the lumen is not responsible for the impermeability of the P cell apical membrane. These drugs completely abolish fluid transport by the Malpighian tubules (Berridge, 1966). Furthermore, after incubation in Ringer's solution without K+ no uptake of luminal dye by the P cells was noticed. Fluid transport through the P cells should be strongly inhibited in the absence of K+ (Berridge, 1968). The experiments with nigericin and some inhibitors of the mitochondrial membrane potential indicate that Rh 123 was indeed accumulating in the S cell mitochondria.

Experiments using the K+ /H+ ionophore nigericin prior to injection of Rh 123 into the lumen showed a strong inhibition of dye accumulation by the S cells, although their mitochondrial membrane potential should be increased by this treatment (Johnson et al., 1981). In cultured vertebrate cells, no inhibition of dye uptake by nigericin was found (Johnson et al., 1981). Monensin (a Na+/H+ ionophore) on the contrary does not inhibit the uptake of Rh 123. These two ionophores cause a non-electrogenic dissipation of Na+ or K+ gradients, with a concomitant increase or decrease in cellular pH. So altering intracellular K+ concentration and/or pH with nigericin seems to inhibit the uptake of rhodamine 123, although the mitochondrial membrane potential is increased. The very specific (electrogenic) potassium ionophore valinomycin also inhibits the dye uptake, but this ionophore also dissipates the electrochemical gradient across the mitochondrial membranes (Johnson et al., 1981).

No inhibition of accumulation of Rh 123 by the S cells was found with ouabain (10^-4 M). Atzbacher et al. (1974) described inhibitory effects of ouabain on the excretion of acidic dyes in Drosophila, and ouabain-sensitive ATPases have been characterized in some insect Malpighian tubules (Bradley, 1985). The uptake of Rh 123 by the S cells was also not inhibited by the absence of extracellular Na+ or K+. Bresler et al. (1985, 1990) described Na+-dependent fluorescein transport in the Malpighian tubules of several insects. Rafaeli-Bernstein and Mordue (1979) found no inhibitory effect of very low sodium concentrations on glucose reabsorption in Locusta Malpighian tubules, although according to these authors this transport should be sodium-dependent.

The process of accumulation by the S cells is quite fast. This might be an indication of facilitated diffusion of Rh 123 through the apical S cell membrane. Possibly the S cells contain carrier proteins in their apical membrane that facilitate passage into the S cells. Rather non-specific carriers have been described for the transport of organic acids and bases in the vertebrate kidney tubules (Ullrich, 1979) and for organic acids in insect Malpighian tubules (Bresler et al., 1985, 1990). This transport is believed to be carrier-mediated, since it was completely inhibited by competitive inhibitors. Strictly speaking, the transport of organic bases in the mammalian proximal kidney tubules is not active, since it represents facilitated diffusion that is largely dependent on the cell membrane potential and thus on cellular metabolism (Ullrich, 1979).

The mechanism for selective accumulation by the S cells remains unknown, but the impermeability of the apical P cell membrane might play an important role in this process. If the apical P cell membrane is largely impermeable, then Rh 123 can only diffuse into the S cell. In the distal parts of the posterior tubules, little accumulation by the S cells is noted, while the P cells take up the Rh 123 in these parts. When after some time the P cells start loosing the dye in this part, the S cells accumulate the dye from the lumen. The mitochondrial membrane potential seems to be the major driving force for this accumulation. Since Rh 123 only labels the S cells well when presented at their apical membrane, some process at this membrane must facilitate the entry into the cell, or the basal cell membrane must be quite impermeable to the dye. The specific inhibition of uptake after incubation in nigericin also indicates some process at the apical membrane that might facilitate entry into the S cell. The lack of information concerning ion transport phenomena in the secondary cell type makes it difficult to explain the accumulation by the S cells.

Also of interest is the apparent regionalisation of the morphologically uniform PMT, at least in relation to the release and uptake of Rh 123. Maddrell (1978b) also
No evidence was found for endocytosis of the fluorescently labeled dextrans, fluorescently labeled bovine serum albumin or Lucifer Yellow CH from the Malpighian tubule lumen. This of course does not exclude any endocytosis of other molecules, but it strongly suggests that there is no bulk pinocytosis of luminal contents. The speed with which Rh 123 accumulates in the S cells also indicates a non-endocytotic process. Vigorous endocytosis of luminally injected dextrans has been described in mammalian kidney tubules (Schwartz and Al-Awqati, 1990).

Many potassium-transporting insect tissues like the midgut and the Malpighian tubules contain (apical) membrane-bound particles (Gupta and Berridge, 1966; Berridge and Oshman, 1969). These ‘portasomes’ were first described as apical K⁺-transporting pumps (Harvey et al., 1983). Finally, the potassium transport in lepidopteran gobot cells was found to depend upon apical proton ATPases (Wiezorek et al., 1989, 1991). These vacuolar-type proton ATPases were found to provide the energy for potassium transport out of the cell, by means of K⁺/H⁺ antiport. Although in most animal cells Na⁺ gradients are used as an energy source for organic ion transport, several proton-gradient-based organic ion transport systems have been found in animal cells. Ganapathy and Leibach (1991) described proton-coupled solute transporter in different vertebrate cell types, among which is an organic cation transporter. This transport system would be driven by a proton gradient, using both the chemical and electrical components of this proton gradient as an energy source. In the brush border of renal proximal tubule cells, Na⁺/H⁺ exchange would provide the energy for the transport of the cation N-methylisocyanamide (NMN) by creating a H⁺ gradient (Ross and Holohan, 1983). Vacuolar-type proton ATPases generate the electrochemical gradient that permits the accumulation of neutral or positively charged biogenic amines into acidic vesicles (Mellman et al., 1986). Possibly the organic cation Rh 123 could use similar routes for entering the S cells, by a possible combination with H⁺-ATPase. However, it would seem unlikely that the positively charged (and permeant) dye molecule would need such a system to enter the (negatively charged) S cell’s cytoplasm. More probably, any Rh 123 entering the P cells could be returned to the lumen by means of such a system. This system could eliminate cationic molecules via the urine under normal circumstances. However, no evidence of the short-circuiting of such a mechanism by means of proton ionophores was found in this study.

Recently, Bertram et al. (1991) described the inhibition of in vitro fluid secretion in Drosophila Malpighian tubules by bafilomycin A₁ and other inhibitors of vacuolar-type ATPases. Leyssens et al. (1991) found evidence for an electrogenic proton ATPase at the apical membrane of Formica Malpighian tubules. The same group described an alkalinisation of intracellular pH upon stimulation of KCl and fluid secretion (Zhang et al., 1991). So evidence is accumulating to suggest that the Malpighian tubules of insects secrete primary urine
by means of proton ATPase-dependent cation (K\(^+\)) transport. Recently, Klein et al. (1991) have even localised the vacuolar proton ATPase in the apical membranes of midgut and Malpighian tubules of *Manduca sexta* moths. According to the authors, these Malpighian tubules only comprise one type of cell. Such plasma membrane proton ATPases were also detected in the K\(^+\)-transporting insect sensilla by means of monoclonal antibodies (Klein and Zimmermann, 1991). Further studies will have to be focussed on the presence and possible involvement of vacuolar-type ATPases in the reabsorption and/or exclusion of organic cations.

We have already indicated the suitability of the fluorochrome rhodamine 123 as a selective supravital label for the secondary cell type in the Malpighian tubules of *Sarcophaga bullata* (Meulemans and De Loof, 1991). This also seems to hold for the intravital labeling of the other Diptera (Brachycera) examined. Such selective vital labeling may facilitate future research on these relatively unknown secondary cells. This dye also clearly shows the polarity of the different Malpighian tubule cells. In the P, as well as in the S cells, differences in intracellular fluorescence intensity are found after application of the dye to the apical or basolateral cell membranes. As well as facilitated transport processes, the possible influence of extracellular (charged) molecules of the cell's glycoalyx on the differential permeability of membranes should not be overlooked (Gupta, 1989).

Berridge and Oschman (1969) suggested that the S cells in *Calliphora* could reabsorb sodium from the lumen (hypothetically), while Taylor (1971) described similar cells in *Carausius* and other insects as mucocytes because of their positive reaction for acid mucopolysaccharides. In many insect Malpighian tubules cells with similar morphology to the secondary cells of Diptera are found (Martoya and Ballan-Dufraisne, 1984). Whether they also exhibit similar characteristics remains to be investigated. Principal and intercalated cell types have also been distinguished by means of a mitochondria-selective (carbocyanine) dye in the mammalian kidney tubules (Schwartz and Al-Awqati, 1990). This differential staining seemed to be based upon differences in numbers of mitochondria rather than on differences in membrane permeability or dye retention. Cells with opposite polarities of H\(^+\) secretion have been described in the kidney collecting duct (Schwartz et al., 1985).

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